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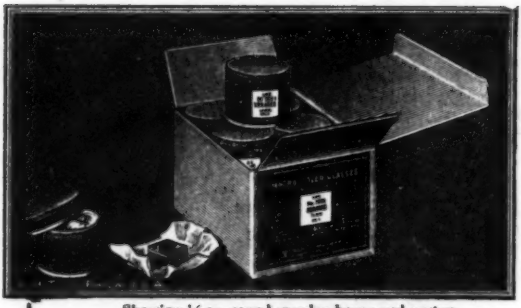

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CELL DIVISION INHIBITION OF ARBACIA AND CHAETOPTERUS EGGS AND ITS REVERSAL BY KREBS CYCLE INTERMEDIATES AND CERTAIN PHOSPHATE COMPOUNDS [1, 2]

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It has been demonstrated that anoxia (Loeb, 1895), cyanide (Lyon, 1902), azide (Clowes and Krahl, 1939), and dinitrophenol (DNP) (Clowes and Krahl, 1936) inhibit the cleavage of *Arbacia punctulata* eggs. These metabolic inhibitors are known to interfere with hydrogen transport (Stannard and Horecker, 1948) and/or phosphorylation (Reiner and Spiegelman, 1947). Because "high energy phosphate" ($\backsim$P) is equilibrated with the adenosine triphosphate (ATP) within the cell (Green and Colowick, 1944), it was considered possible that the inhibition of cleavage, produced by the above agents, might be reversed by addition of ATP.

Further, because current ideas relate the oxidative formation of $\backsim$P to the functioning Krebs cycle (Lipman, 1941), inhibition of the cycle by malonate might inhibit cleavage because of $\sim$P lack. If the general scheme of $\sim$P generation applied to *Arbacia,* relief of the malonate division inhibition would be effected by succinate, a substance which competes with malonate for succinic dehydrogenase; fumarate, an intermediate of the Krebs cycle beyond the point of action of malonate (Potter and Dubois, 1943); and ATP, the postulated critical product of the cycle (Lipman, 1946).

Two reasons prompted an extension of the experiments to include *Chaetopterus,* as well as *Arbacia,* eggs: (1) *Arbacia* eggs show an increase, and *Chaetopterus* eggs a decrease, in oxygen consumption at the time of initiation of cell division (Whitaker, 1932); (2) if *Arbacia,* an Echinoderm, and *Chaetopterus,* an Annelid, exhibit similar reactions to malonate, it could indicate a general importance of the Krebs cycle and ATP to cell division in aerobic organisms.

[1] The author would like to express his sincere gratitude to Dr. J. O. Hutchens, Dr. D. L. Harris and Dr. J. Z. Hearon for their encouragement and constructive criticism. Dr. G. H. A. Clowes was kind enough to make the Eli Lilly Laboratories at Woods Hole available for part of this work. Grateful acknowledgment is made to Manfred Brust for technical assistance.

[2] Completed in part while Fellow of the American Cancer Society (recommended by the Committee on Growth of the National Research Council).

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Methods

Arbacia punctulata and *Chaetopterus pergamentaceus* eggs were washed in sea water and adjusted to 0.1 to 0.2 per cent by volume by taking a small aliquot of the egg suspension and centrifuging it at 1000 times gravity in duplicate hematocrit tubes. From the egg volume the number of eggs present in each experimental vessel was calculated (Harvey, 1932; Krahl, 1950). A second aliquot of eggs was fertilized and the per cent cleavage noted at the end of one hour as a test of both egg and sperm viability before use. Egg suspensions were used only if they showed 90 to 100 per cent cleavage. The fertilized eggs were added to the experimental vessels after 25 minutes (*Arbacia*) or 40 minutes (*Chaetopterus*).

In low oxygen tension experiments, mixtures of air and nitrogen were prepared by displacement of water in a calibrated glass bottle (Umbreit *et al.*, 1949). The bottle was connected by rubber tubing to serially arranged double-arm Warburg vessels which contained, in the main compartment, fertilized eggs and reversing agent or NaCl. The rate of gassing was maintained approximately constant by regulating the rate of bubble evolution by means of a screw clamp placed at the end of the last tube which dipped below the surface of the water bath. The gas mixture was passed through the serially connected vessels for 30 minutes with shaking at an amplitude of six centimeters and rate about 120 per minute. At the end of the gassing period the vessels were closed and the shaking continued for an additional two hours. The control differed from the experimental vessels only in the substitution of air for the gas mixture. The disadvantage of a serially connected system is the possibility of unequal gassing of the individual flasks. To minimize this disadvantage, the order of the flasks was varied from experiment to experiment. To insure adequate removal of air, the volume of flushing gas used was 20 times the volume of the empty system.

When azide, cyanide, and DNP[4] were used as division-inhibiting agents, the experiments were conducted in air-filled closed vessels to which 3.3 mg. per ml. of glycylglycine buffer was added at pH 8.2. No buffer was required with malonate and its reversing agents, succinate and fumarate, because the latter solutions were adjusted to pH 8.2 with solid sodium hydroxide. Two types of experiments were performed when malonate was used to inhibit cell division. In some experiments solid sodium hydroxide was added to malonic acid dissolved in sea water which produced a hypertonic solution. In other experiments solid sodium hydroxide was added to malonic, fumaric or succinic acid dissolved in distilled water to produce a solution isotonic with sea water. Experimental temperature varied between 22–27° C., but within a single experiment the temperature was constant to ± 0.1° C.

In all instances at the conclusion of the experiment (two to three hours) egg cleavage was stopped with formaldehyde and the number of divisions per egg in each vessel was calculated according to the method of Smith and Clowes (1924). In this calculation 100 eggs are counted and the cleavage stage of each egg is recorded as follows: no divisions, one cell; one division, two cell; two divisions, four cell; etc. The sum of the number of divisions divided by 100 yields a number designated as the number of divisions per egg.

[4] The DNP was a purified sample obtained through the kindness of Dr. G. H. A. Clowes of the Eli Lilly Laboratories, Woods Hole, Mass.

Experimental Results

Interference with the hydrogen transport system near its terminus

Graphically presented in Figure 1 are the results of typical experiments in which low oxygen tension was used to inhibit cleavage of *Arbacia* eggs. It is seen that in all instances ATP stimulated cell division of the inhibited eggs. Further, the added ATP completely reversed the division inhibition provided the latter was not greater than 50 per cent. Figure 2 shows that adenylic acid (AA),

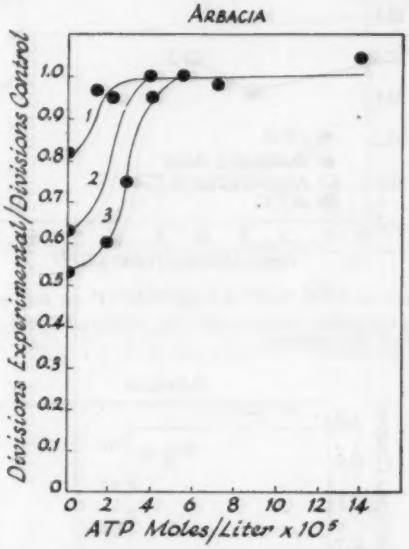

FIGURE 1. The reversal effect of ATP on low oxygen tension-inhibited *Arbacia* eggs. Rate of oxygen consumption as per cent of control: (1) 75; (2) 60; (3) 50. Experimental duration 100 minutes.

hydrolyzed ATP (HATP), or the ~ P compound, inorganic triphosphate (ITP), were not effective in reversing the division inhibition.

Because added ATP completely reversed low oxygen tension inhibition of *Arbacia* egg cleavage, it was thought a similar reversal might be obtained when cyanide or azide were used as inhibiting agents. The results with sodium azide are presented in Figure 3. The results with sodium cyanide are presented in Figure 4. It is seen that the inhibition of cleavage in *Arbacia* eggs is:

(1) Reversed when ATP is added to cyanide-inhibited cells.
(2) Not reversed when ATP is added to azide-inhibited cells.
(3) Not reversed when hydrolyzed ATP is added to either cyanide- or azide-inhibited cells.

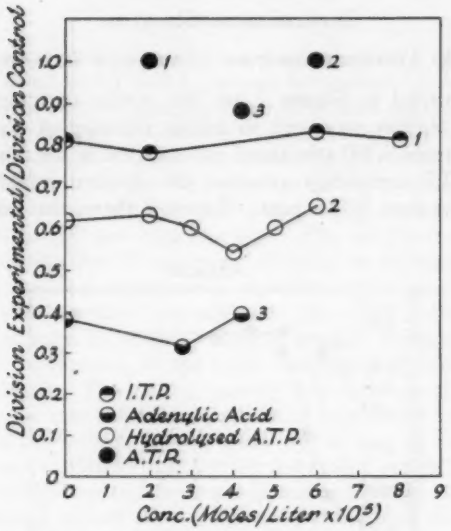

FIGURE 2. The effect of ATP, ITP, AA and HATP on low oxygen tension—inhibited *Arbacia* eggs. The corresponding numbers are the results obtained with the same batch of eggs. Experimental period 100 minutes.

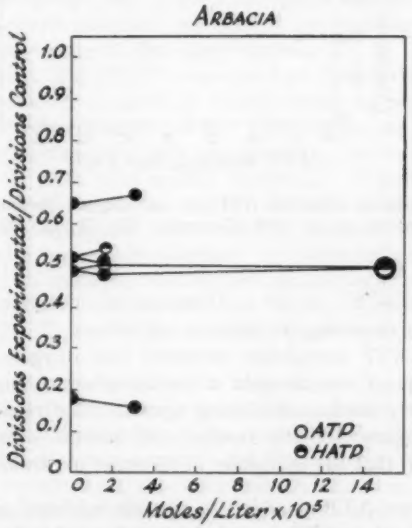

FIGURE 3. The effect of ATP and HATP on azide-inhibited *Arbacia* eggs. With increasing inhibition the concentration of NaN_3 was: 1.35, 1.25, 2.7×10^{-5} *M* sodium azide, and the experimental time was 180, 100 and 180 minutes, respectively.

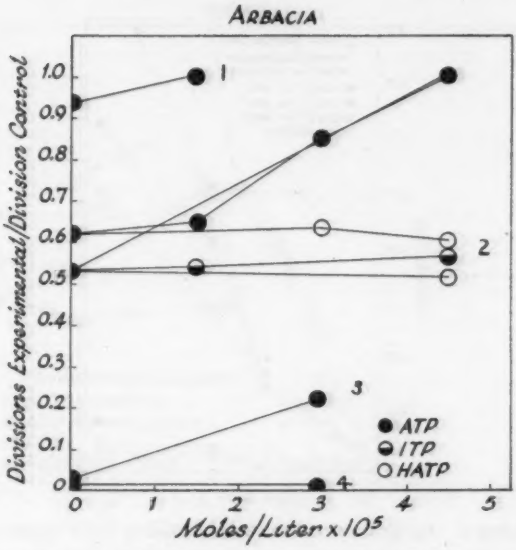

FIGURE 4. ATP, ITP and HATP effect on cyanide-inhibited *Arbacia* eggs. Cyanide concentration (1) 2; (2) 6; (3) 8; (4) 16×10^{-5} *M*. Experimental time 100 minutes.

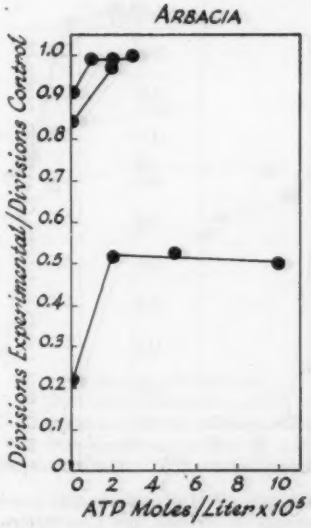

FIGURE 5. ATP effect on DNP-inhibited *Arbacia* eggs. Concentration of DNP with increasing amount of inhibition: 0.4, 0.8 and 1.6×10^{-5} *M*. Experimental time 100 minutes.

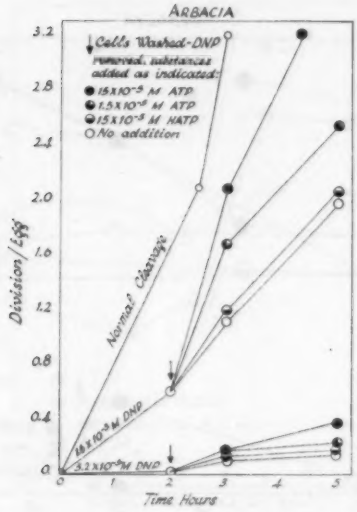

FIGURE 6. Recovery of *Arbacia* eggs following DNP inhibition.

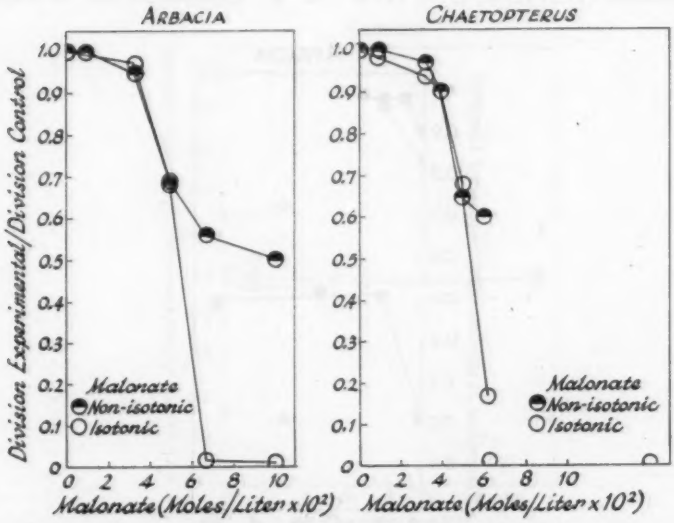

FIGURE 7. The inhibitory effect of isotonic and non-isotonic malonate on the cleavage of *Arbacia* and *Chaetopterus* eggs.

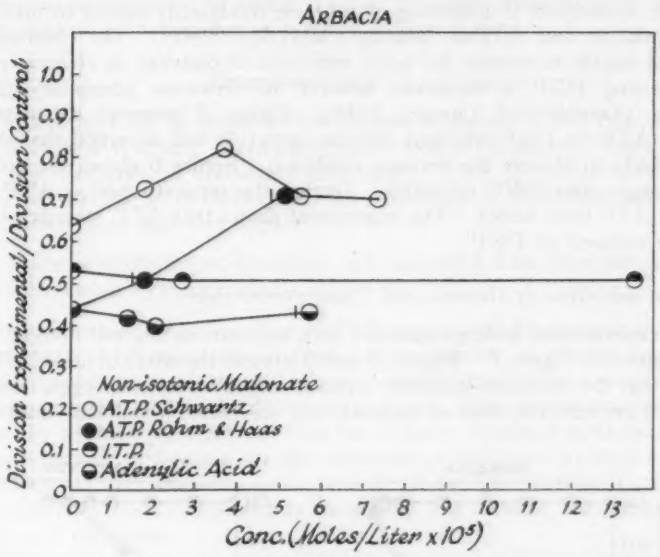

FIGURE 8. The effect of ATP, ITP and AA on non-isotonic malonate-inhibited *Arbacia* eggs. Experimental time 100 minutes.

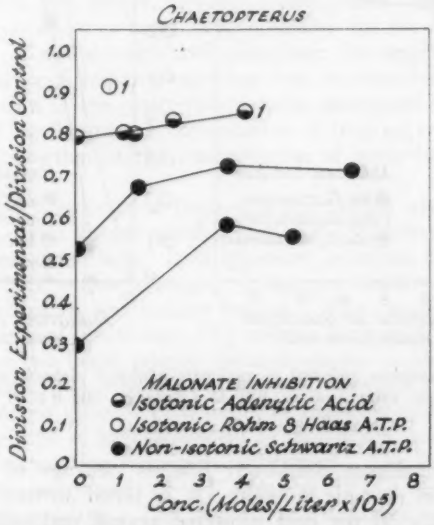

FIGURE 9. ATP and AA effect on isotonic and non-isotonic malonate-inhibited *Chaetopterus* eggs. The number indicates same experiment and same batch of eggs. Experimental time 100 minutes.

Azide, in addition to inhibiting cytochrome oxidase, is known to inhibit transphosphorylation and ATPase activity (Meyerhof, 1945). The observation that ATP was unable to reverse the azide inhibition of cleavage in *Arbacia* eggs suggested testing DNP, a substance believed to dissociate phosphorylation from oxidation (Loomis and Lipman, 1948). Figure 5 presents the data of the effect of ATP on DNP-inhibited *Arbacia* eggs. It will be noted that ATP was able partially to reverse the division inhibition. Figure 6 shows the recovery of *Arbacia* eggs after DNP inhibition. During the recovery period ATP and hydrolyzed ATP were added. The experiment shows that ATP stimulated cleavage after the removal of DNP.

Malonate inhibition of Arbacia and Chaetopterus eggs

The experimental findings obtained with malonate as the cell division inhibitor are presented in Figure 7. Figures 8 and 9 present the effect of added ATP, ITP, and AA on the malonate-inhibited *Arbacia* and *Chaetopterus* eggs, respectively. Figure 10 presents the effect of fumarate and succinate on malonate-inhibited eggs.

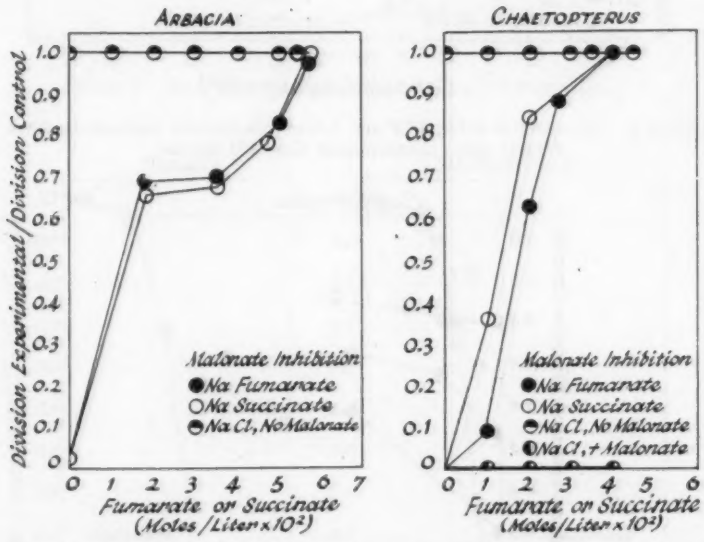

FIGURE 10. The complete reversal of malonate-inhibited *Arbacia* and *Chaetopterus* eggs by succinate and fumarate using isotonic 10^{-1} *M* (*Arbacia*) and 6×10^{-2} *M* isotonic malonate (*Chaetopterus*).

It is seen that malonate completely inhibits cleavage of both *Arbacia* and *Chaetopterus* eggs in isotonic solution. It is noted further that *Arbacia* and *Chaetopterus* eggs are 50 per cent protected against malonate by hypertonicity. At the concentrations tested, succinate and fumarate completely, and ATP incompletely, reverse the malonate inhibition. Complete reversal of the malonate

inhibition was not obtained by added ATP at the concentrations which completely reversed division inhibition due to reduced oxygen tension.

Discussion and Conclusions

Sea urchin eggs fail to divide at low oxygen tensions. From this observation Loeb (1895) and Warburg (1914) postulated that extra energy, dependent upon oxygen, was necessary for division of these eggs. Thus, if oxidative energy is involved in cell division of *Arbacia* eggs, then a decrease in the oxygen tension could inhibit division in at least two ways:

(1) Decrease in hydrogen transport, not associated with phosphorylation.
(2) Decrease in ~ P formation.

An experiment performed by Korr (1939) suggests that a decrease in hydrogen transport *per se* is not primarily involved in the inhibition of *Arbacia* egg division. He added cyanide to respiring *Arbacia* eggs which resulted in a decrease in the oxygen consumption and an inhibition of division. The respiration was restored to normal by addition of methylene blue but division remained inhibited. Methylene blue is known to by-pass the dehydrogenase-cytochrome hydrogen transport system which has been shown to generate ~ P (Lehninger, 1949). Consequently, these results have been interpreted as indicating that division fails because of insufficient ~ P formation.

Whether reduced oxygen tension is able to decrease ~ P generation and subsequently inhibit cell division depends upon the relative amount of ~ P formed aerobically and anaerobically by the cell. For example, frog eggs show a very high rate of glycolysis (Barth and Jaeger, 1947). Reduction of the oxygen consumption of these eggs by cyanide or reduced oxygen tension has no effect upon division or the concentration of easily-hydrolyzed phosphate (interpreted as ~ P). Addition of azide to the respiring frog eggs not only reduces the oxygen consumption and the concentration of the easily-hydrolyzable phosphate but also inhibits division. Thus, reducing the oxygen consumption of frog eggs has no effect on division; whereas, a reduction of the concentration of easily-hydrolyzable phosphate inhibits their division.

Unlike frog eggs, division of *Arbacia* eggs is inhibited when the oxygen tension is reduced, and it is not known whether the ~ P concentration of the egg-cell is reduced under these conditions. It is apparent, however, from results here presented that intact ATP is able to support division of *Arbacia* eggs when their division has been inhibited by low oxygen tension. These results are indicative that division failed because of insufficient ATP and/or substances or systems dependent upon ATP. There is a linear relation between oxygen tension and division in the range where oxygen tension influences division (Clowes and Krahl, 1939). Hence, if division is dependent upon ~ P, it might be expected that the amount of ATP added as ~ P would be at least equivalent to that which would have been formed had the oxygen consumption remained undisturbed. The following simple calculation shows that the micromoles of ~ P added as ATP is approximately ten times that calculated to be required. This calculation constitutes no proof that the ATP added was used as an energy source necessary for division. Further experiments are in progress to evaluate this point.

Normal dividing and respiring *Arbacia* eggs consume 2.4 mm.^{3} oxygen per 10 mm.^{3} egg hours (Hutchens *et al.*, 1942). Because 46,500 eggs occupy a volume of 10 mm.^{3} (Harvey, 1932), the oxygen consumed per egg hour is 5.2×10^{-5} mm.^{3}. Division is normal and 50 per cent inhibited when the oxygen consumption of the *Arbacia* egg is reduced to 80 per cent and 50 per cent, respectively (Clowes and Krahl, 1939). Thus, 1.42×10^{-6} microatoms of oxygen per egg hour is the difference between the amount of oxygen consumed by normal and 50 per cent division-inhibited eggs. This oxygen consumption difference may be converted to micromoles of $\sim$ P on the assumption that three micromoles of $\sim$ P are produced per microatom of oxygen consumed (Ochoa, 1943). The result is 4.26×10^{-6} micromoles of $\sim$ P per egg hour.

The amount of ATP added to 930 eggs was 4.1×10^{-5} mM, or 2.05×10^{-5} mM of ATP per egg hour for the two-hour experimental period. ATP contains two $\sim$ P bonds (Lohman, 1938). Therefore, the amount of $\sim$ P added is 4.4×10^{-5} micromoles per egg hour as compared with 4.26×10^{-6} micromoles $\sim$ P per egg hour calculated from the oxygen consumption difference. These calculations indicate that the micromoles of $\sim$ P added as ATP are approximately ten times the amount calculated to be required.

The calculation presented is at least consistent with the concept that ATP acts to supply $\sim$ P to anoxia-inhibited eggs. However, that something in addition to ATP is necessary for division is indicated by the experiments in which ATP was added to malonate- and DNP-inhibited eggs. In both these instances, added ATP stimulated the inhibited eggs, but in no instance was the division rate re-established to normal. Greater reversal might have been obtained had higher concentrations of ATP been used. However, Runnström and Kriszat (1951) found a maximum of 40 per cent reversal when 100 times the concentration of ATP was added to DNP-inhibited sea urchin eggs.

It has been suggested (Runnström and Gustafson, 1951) that ATP could affect cell division by influencing cytoplasmic viscosity, similar to the effect of ATP on actomyosin threads and solutions. It is well known (Heilbrunn, 1943) that at the time of division there is a marked decrease in protoplasmic viscosity, and Runnström and Kriszat (1950) have shown that ATP added to unfertilized eggs decreases the cellular cytoplasmic viscosity. To test the possibility that added ATP might function in cell division as a substrate for an actomyosin-like protein, ITP, Pyro. phosphate (Pyro. phos.), and ATP were added to division inhibited eggs. These reagents have been shown to contract actomyosin threads and to decrease the viscosity of actomyosin solutions (Biro and Straub, 1949). As previously noted, of these reagents only ATP was effective in promoting division of the inhibited eggs. These results may indicate: (1) that ITP and Pyro. phos. were not accessible to the division mechanism; (2) that the egg actomyosin-like protein does not react with these substances; or (3) that the function in division of added ATP is not to support an actomyosin-like reaction. Further experiments are in progress to evaluate these points.

To summarize the results presented, the action of an inhibitor which interferes with hydrogen transport (cyanide and low oxygen tension) can be completely reversed by added ATP, provided the inhibition is not greater than 50 per cent. If either ATP utilization or the generation of metabolic intermediates are inter-

fered with (azide and DNP), ATP alone cannot make up the deficit. The fact that malonate is able completely to inhibit division of *Arbacia* and *Chaetopterus* eggs, and succinate and fumarate are able completely to reverse this inhibition, is strong evidence that division is intimately dependent upon a functioning Krebs cycle. Further, because ATP was unable to reverse completely the division inhibition due to the action of malonate, the function of the Krebs cycle in division may not be completely accounted for on the basis of $\sim$ P generation.

Summary

Division of *Arbacia* eggs was inhibited by low oxygen tension, cyanide, azide, dinitrophenol (DNP) and malonate. Various high and low energy phosphate compounds, and succinate and fumarate were added to the inhibited eggs. The results were extended to malonate-inhibited *Chaetopterus* eggs and the effects of succinate and fumarate were noted.

1. Addition of 10^{-5} to 10^{-4} *M* ATP completely reversed *Arbacia* egg division inhibition produced by cyanide and low oxygen tension. ATP incompletely reversed inhibitions caused by DNP and malonate. The maximum division stimulations were: with low oxygen tension and cyanide, division was increased from 50 per cent to 100 per cent of the control value; with 1.6×10^{-5} *M* DNP, division was increased from 23 to 51 per cent; with 7.5×10^{-2} *M* malonate, division was increased from 44 per cent to 71 per cent. With *Chaetopterus* eggs, the maximum stimulation obtained in the presence of 0.1 *M* malonate was from 28 per cent to 58 per cent of the control value.

2. Adenylic acid, hydrolyzed ATP, inorganic phosphate, and the $\sim$P compounds, inorganic triphosphate and pyrophosphate, did not stimulate division of *Arbacia* eggs when inhibited by any of the above agents.

3. Division of *Arbacia* and *Chaetopterus* eggs was completely inhibited by 10^{-1} to 10^{-2} *M* malonate. The malonate effect was completely reversed, in both egg types, by the addition of 5.7×10^{-2} *M* succinate or fumarate.

4. Azide inhibition was not relieved by added ATP or any of the other phosphorylated compounds tested.

5. These results suggest, in *Arbacia* and *Chaetopterus* eggs, that division is intimately related to a functioning Krebs cycle, the function of which cannot be completely accounted for on the basis of $\sim$ P production.

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ENDOCRINE CONTROL OF METABOLISM IN THE LAND CRAB, GECARCINUS LATERALIS (FRÉMINVILLE). I. DIFFERENCES IN THE RESPIRATORY METABOLISM OF SINUSGLANDLESS AND EYESTALKLESS CRABS[1,2]

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Long ago eyestalk removal was observed by Zeleny (1905) to induce molting in *Uca* and by Megušar (1912) to reduce the length of the intermolt interval of *Astacus*. Many years later Abramowitz and Abramowitz (1938, 1940), Hanström (1939), Brown and Cunningham (1939), Smith (1940) and Kleinholz and Bourquin (1941) also reported compression of the intervals between successive molts in a variety of eyestalkless crustaceans. Revival of interest in the crustacean eyestalk as a molt-inhibiting center may be attributed to the discoveries in the meantime that the eyestalk contained (1) chromatophorotropically active materials (Koller, 1925, 1927; Perkins, 1928), and (2) two organs, the sinus gland and the x-organ, which gave cytological evidence of secretory activity (Hanström, 1931, 1933). Subsequent studies, primarily by Brown and his students (see Brown, 1935, 1940), yielded considerable evidence favoring the sinus gland as the principal source of these chromatophore-activating materials.

The activity of the sinus gland did not seem to be confined to chromatophore regulation. Kleinholz (1934, 1936) clearly demonstrated the response of crustacean retinal pigments to eyestalk extract, thus confirming the suggestions of Welsh (1930a, 1930b) that there is hormonal control of these pigments. Later work by Welsh (1939, 1941) pointed directly toward the sinus gland as the agent responsible. On the basis of evidence offered by Brown and Cunningham (1939), Scudamore (1942, 1947), Kyer (1942) and Bauchau (1948a), molting was added to the list of processes thought to be controlled by the sinus gland.

It was realized, however, that since the concept of hormonal regulation by the sinus glands was based to a large extent upon effects of total eyestalk removal, the critical experiment remained to be done, namely, removal of sinus glands alone. A method devised by Brown (1942) produced in sinusglandless crayfish certain responses similar to those which characterize eyestalkless animals. A removal technique, by which injury to other eyestalk tissues was minimized, was designed and employed by Kleinholz (1947). He found (1948, 1949a, 1949b) that the

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[2] These materials constitute a portion of a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree from Radcliffe College.

proximal pigments of the crayfish retina behaved normally after sinus gland removal. Extra-sinusglandular sources of retinal pigment hormone had not been conclusively demonstrated, since there remained the possibility, according to Kleinholz, that the pigments could also act as independent effectors. Nevertheless, here was an indication that, in one respect at least, a crustacean without its sinus glands could still be normal.

With the application by recent workers of the Kleinholz sinus gland removal technique or its various modifications, the mystery of the sinus gland has been clarified. Most of this work has been reported only in abstracts (Bliss, 1951; Frost, Saloum and Kleinholz, 1951; Havel and Kleinholz, 1951; Passano, 1951a, 1951b, 1952a; Travis, 1951b; Welsh, 1951) or in unpublished theses (Frost, 1948; Havel, 1949; Saloum, 1951; Travis, 1951a; Bliss, 1952; Passano, 1952b). The present paper summarizes physiological evidence in support of morphological observations and interpretations already presented (Bliss and Welsh, 1952). Each sinus gland of a crab should now be conceived as a storage-release center for secretory material synthesized within cells of a diffuse neurosecretory system, involving the eyestalk ganglia, the brain and perhaps the thoracic ganglionic mass. Metabolic data obtained by Kleinholz and co-workers, coupled with morphological observations by Mr. James B. Durand, Jr. of Harvard University (personal communication), suggest that a similar relationship, demonstrable in crayfish, characterizes the Macrura as well.

Materials and Methods

Specimens of *Gecarcinus lateralis* (Fréminville) were available in large numbers in Bermuda where these studies were commenced. During the major part of the work specimens were shipped periodically to Cambridge from Bermuda. Laboratory stocks were maintained at room temperature in sand-filled cement tanks, divided by wire partitions into sections suitable for 15 to 20 crabs each. During observation of a given crab, the animal was isolated in a rectangular gallon glass jar to which had been added sand and a small finger bowl or, in the case of eyestalkless crabs, a Petri dish of sea water or tap water. The crabs were observed to sit in the water and occasionally, by use of a large claw, to drink. Peanuts, lettuce and carrots were accepted by the crabs, peanuts being preferred. Mortality both during air shipment and during maintenance was very low.

Surgery

The crab was cooled in the refrigerator for about an hour before sinus gland removal. It was then supported by plasticene in a cardboard holder which in turn lay, surrounded by ice, in a Büchner funnel. Rubber sheeting was laid over the crab's back and more ice was placed on the sheeting. The Büchner funnel was so mounted in a wooden table that the funnel top was flush with the table top (see Williams, 1946). Ice melt-water ran from the funnel into a collecting pan beneath the table.

Eyestalks were ligatured with coarse surgical cotton thread. By means of a dental burr (1.5 mm. diameter), which was mounted in a handpiece attached to a foot-regulated rheostat, a hole was drilled in the mid-dorsal portion of the eye-

stalk as it lay in its orbit. After the hypodermis had been moved aside and the connective tissue sheath slit, the blue-white sinus gland was extracted by watchmakers' forceps and a small piece of plastic cover slip was sealed with paraffin into the hole. The ligature was then released. The second eyestalk was treated in the same manner.

Eyestalk removal was also preceded by cooling of the crab in the refrigerator and followed by packing of the eyestalk stub with fibrin foam (Cutter Laboratories) or Gelfoam (Upjohn Company). Mortality after either operation was negligible when the crabs were cooled sufficiently. Eyestalk removal without previous cold anesthesia was often fatal, as were operations performed when crabs had remained over an hour in the refrigerator.

Measurement of respiratory rate and respiratory quotient

For respiratory measurements use was made of a volumetric respirometer, the design of which was suggested to the writer by Dr. P. F. Scholander. All plastic and metal portions of this instrument were machined by Mr. John R. Andrews of Randolph, Massachusetts. Of similar principle is the respirometer employed by Flemister and Flemister (1951). Figure 1A shows the essential features of the instrument used by the writer.

The edges of a jar (1) of 450 cc. or 250 cc. capacity have been ground with carborundum so that, when greased and clamped, they make an air-tight seal with a ground plastic cover (2), cut from 1/2-inch Lucite. Dead air space in the animal chamber (1) has been reduced by the addition of paraffin (3). The animal chamber is clamped by a quarter-inch brass crossbar (4), which slides up and down on two threaded brass uprights mounted on either side of a Lucite platform (5). The platform is supported by a strip of brass (6). One arm of a plastic manometer (7), designed by Scholander (1949), is connected to the animal chamber. The other arm of the manometer leads to a thermobarometer (8), a bottle having a capacity of 250 cc. and containing a small amount of water. Manometer fluid, devised by Dr. Howard A. Schneiderman of Harvard University, consists of a 1:200 dilution of a concentrated liquid detergent, Aquet (Emil Greiner Company), to which is added three drops of 30% H_2SO_4 and enough acid fuchsin to give a bright red color. Acidification insures that only negligible amounts of carbon dioxide can be taken up by the manometer fluid. In the plastic cover of the animal chamber is a hole, plugged with a vaccine bottle stopper (9) and situated directly over a round, shallow plastic KOH dish (10), which is suspended by a screw from the plastic cover. To facilitate quantitative removal of the alkali, the dish is slightly funnel-shaped, has a small central depression, and is coated with a thin film of paraffin. The two arms of the manometer are open to air. When the instrument is in use, one opening is closed by a solid Lucite plug (11) and the other opening, in the arm leading to the animal chamber, is connected by a hollow Lucite taper (12) and eighth-inch plastic tubing (13) to a greased 10-milliliter hypodermic syringe (14), wired firmly to the animal chamber cover so that when the instrument is in the constant temperature bath, the syringe is under water. This syringe serves as a calibrated oxygen reservoir.

Figure 1B is a diagram of the gas analyzer used in the determination of the amount of carbon dioxide given off by a crab. To a Scholander plastic manometer

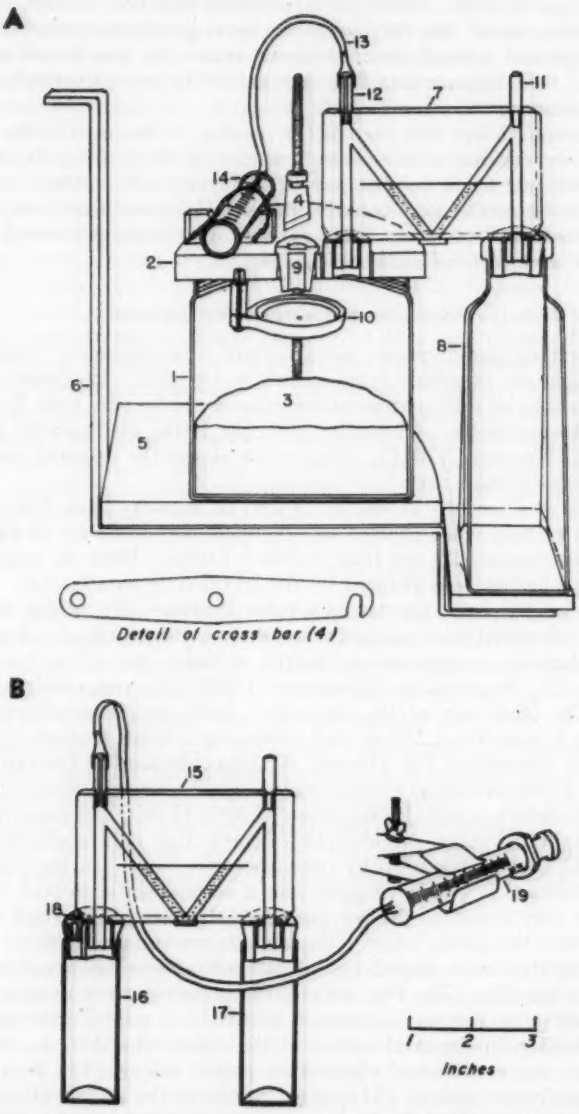

FIGURE 1. (A) Volumetric respirometer and (B) gas analyzer for volumetric determination of carbon dioxide, both drawn as if sectioned longitudinally through the midline. For explanation of figure numbers, see text.

(15) are attached two 18-milliliter glass shell vials, a dry vial (16) to act as a reaction vessel and a moistened one (17) to serve as a thermobarometer. In the rubber stopper of the reaction vessel is a small vaccine bottle stopper (18) and from the reaction vessel an arm of the manometer leads by means of plastic tubing to a 10- or 20-milliliter hypodermic syringe (19) which acts as the calibrated carbon dioxide measuring device. The manometer and shell vials are clamped in a constant temperature water bath and the carbon dioxide syringe is secured alongside the tank.

Procedure was standardized as follows: A crab was placed in the animal chamber, the instrument was assembled and clamped in the water bath, and the animal chamber was connected to the oxygen reservoir. Two milliliters of 10% KOH were injected into the shallow plastic dish through the vaccine bottle stopper just above it. After an equilibration period of 1½ to 2 hours, the KOH was removed by a hypodermic syringe, the needle point of which had been ground to a flat surface. The KOH was measured and discarded, the dish was rinsed with two milliliters of distilled water which was then quantitatively removed, and two milliliters of 10% KOH, which had been equilibrated to bath temperature (25.1° C.), were added. Immediately the first reading on the oxygen syringe was recorded. Readings to the nearest 0.05 of a milliliter were made at convenient intervals of from 10 to 45 minutes for a total time of 2½ to 4 hours. Most runs lasted about three hours and all runs were arranged to come in the afternoon, so that diurnal variations could be minimized. At the end of the run the KOH was quantitatively removed by a hypodermic syringe and injected at once into the reaction vessel of the gas analyzer. Eight-tenths of a milliliter of 30% H_2SO_4 was injected, the instrument was shaken vigorously until deflection of the manometer fluid reached a maximum (about three minutes), and the evolved carbon dioxide was measured on the attached syringe. This value was corrected for the carbon dioxide remaining in solution and for the carbonate originally present in the base. The crab was then removed from the animal chamber and weighed to the nearest tenth of a gram.

A significant portion of the carbon dioxide released from the KOH by acid remains in solution. Empirical data yielding the necessary correction

$$\left(\frac{\text{theoretical } CO_2 - \text{observed } CO_2}{\text{theoretical } CO_2}\right)$$

were obtained by the release within the gas analyzer of different volumes of carbon dioxide from standard carbonate solutions prepared so as to duplicate the carbonate-hydroxide mixtures resulting from an actual volumetric run. These standards were made by adding varying amounts (0.1–1.2 ml.) of 1.786 N K_2CO_3 to sufficient 1.786 N KOH (10% KOH) to give a total fluid volume of 2.0 ml. Gas release was accomplished by the addition of 0.8 ml. of 30% H_2SO_4 by weight (preparation: 20 ml. of concentrated H_2SO_4, 96% pure, plus 80 ml. of distilled water). Forty-eight determinations gave data in per cent which approximate the curve drawn in Figure 2. This curve was actually plotted from theoretical amounts of carbon dioxide calculated from the following formula:

$$V_t = V_o + \frac{V_o \propto V_f}{V_o + V_o},$$

where V_t represents total CO_2, V_o is observed CO_2, V_f is the volume of fluid (2.8 ml.), V_g is the gas capacity of the shell vial (14.2), and α represents the solubility coefficient for CO_2 at 25 degrees C. (0.570). This solubility coefficient was determined from available data (Seidell, 1940) for potassium acid sulfate and sulfuric acid of the same ionic strength as the solution resulting from the reaction between the acid and KOH–K_2CO_3. The solution was calculated to be approximately 3 N for both H_2SO_4 and $KHSO_4$. Although this equation was formulated independently, it is identical, when simplified, with that developed by Scholander, Claff, Andrews and Wallach (1952).

These theoretical and empirical results indicate that, under the stated experimental conditions, the correction which must be applied to an observed volume of carbon dioxide is almost 9% at a volume of 2.5 ml. but decreases to 4% at 24.0 ml. (Fig. 2). It should be emphasized that the position of the curve and therefore the per cent correction vary with temperature, concentration and amount of KOH–K_2CO_3, concentration and amount of H_2SO_4, and gas capacity of the reaction chamber. The carbon dioxide values from which the respiratory quotients

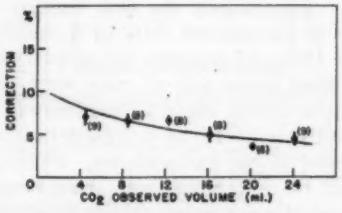

FIGURE 2. Theoretical curve and empirical data showing the correction in per cent which, due to the solubility of carbon dioxide in salt solutions, must be applied to observed volumes when carbon dioxide is evolved under the experimental conditions described in the text. Arithmetic means are indicated by closed circles and standard errors by vertical lines through these circles. Number of determinations indicated in parentheses.

presented in this paper were calculated have been corrected for the amount of carbon dioxide remaining in solution. Consequently, respiratory quotients are somewhat higher than those reported previously (Bliss, 1951, 1952) from the same respiratory data, since at the time of writing of those papers the error had not been recognized and determined.

Blank volumetric runs, performed under conditions identical with those of an experimental respiratory run but without the crab in the animal chamber, revealed that about 1½ hours were required for the respirometer to become equilibrated to the temperature of the water bath. After equilibration had been completed, blank runs of 8 hours' duration produced no measurable deflections of the manometer fluid. As indicated earlier, experimental procedure was standardized to include 1½ to 2 hours of equilibration.

This prolonged period of temperature equilibration served to insure chemical equilibration of the crab's body fluids with the carbon dioxide in transit between the animal and the KOH. A modification of the method described by Scholander, Claff, Andrews and Wallach (1952) was employed in determination of the amount

of carbon dioxide in transit at any given carbon dioxide output rate. Carbon dioxide was released by 0.8 ml. of 30% H_2SO_4 from 2 ml. of KOH–K_2CO_3 in a Stender dish situated within the animal chamber of the respirometer. The volume of evolved gas, about 9.5 ml., was noted on the ten-milliliter greased syringe used for injection of the acid, the base of the syringe needle having, prior to the injection, been sealed into Tygon tubing and pushed firmly into the manometer port leading to the animal chamber. Two milliliters of 10% KOH were injected into the KOH dish. The carbon dioxide uptake by this KOH was followed on the syringe and plotted as a function of time. Tangents drawn to the carbon dioxide uptake curve, as described by Scholander, Claff, Andrews and Wallach (1952), indicated the amount of carbon dioxide in transit. This gas in transit, reaching concentrations within the animal chamber as high as 0.2% of the total gas volume during the respiration of a normal crab and up to 0.7% for a molting eyestalkless crab, can lower the total observed carbon dioxide and secondarily the respiratory quotient (1) by being untrapped in the alkali, and (2) by causing some carbon dioxide to be retained within the buffered body fluids of the crab. For these reasons, in the method described here, the KOH of the equilibration period does not contain the total amount of carbon dioxide produced by the crab during this period. However, this alkali is discarded. The KOH of the experimental run is added after the carbon dioxide of the crab's body fluids, the carbon dioxide in transit, and the carbon dioxide being trapped by the alkali have reached a steady state. Therefore all carbon dioxide produced by a crab during an experimental run is trapped in the KOH added at the beginning of the run. The recorded amount of carbon dioxide evolved from this KOH, when corrected for the gas remaining in solution, is a true measure of total carbon dioxide production during the experiment.

In order that the carbon dioxide buffer capacity of a crab might be estimated, the total oxygen consumption of two normal crabs was recorded first in the presence of KOH and immediately thereafter in its absence. The magnitude of the difference between these amounts, less than expected on the basis of an average respiratory quotient of 0.7 for these crabs as determined by the gas analysis method, was an indication of the power of a crab's body fluids to hold carbon dioxide in chemical combination. Clearly essential to the procurement of valid respiratory data is one of the following: (1) a low tension of carbon dioxide in transit; (2) an extended period of experimental observation and measurement; or (3), as in the method described here, a renewal of the carbon dioxide-absorbing KOH after a prolonged period of equilibration.

During experimental runs either with or without KOH, no sudden deflections of the manometer fluid, indicative of "bursts" of carbon dioxide, were observed. Such "bursts" have been described for insects by Punt (1950) and by H. A. Schneiderman and C. M. Williams (personal communication). The carbon dioxide output of a land crab at 25 degrees C. is steady and continuous. Carbon dioxide output by a land crab, at least at this temperature, can be taken as a true measure of its carbon dioxide production. If the gas is properly trapped by alkali and subsequently released from the alkali and measured, and if the measurement is corrected for solubility error, a valid respiratory quotient can be calculated.

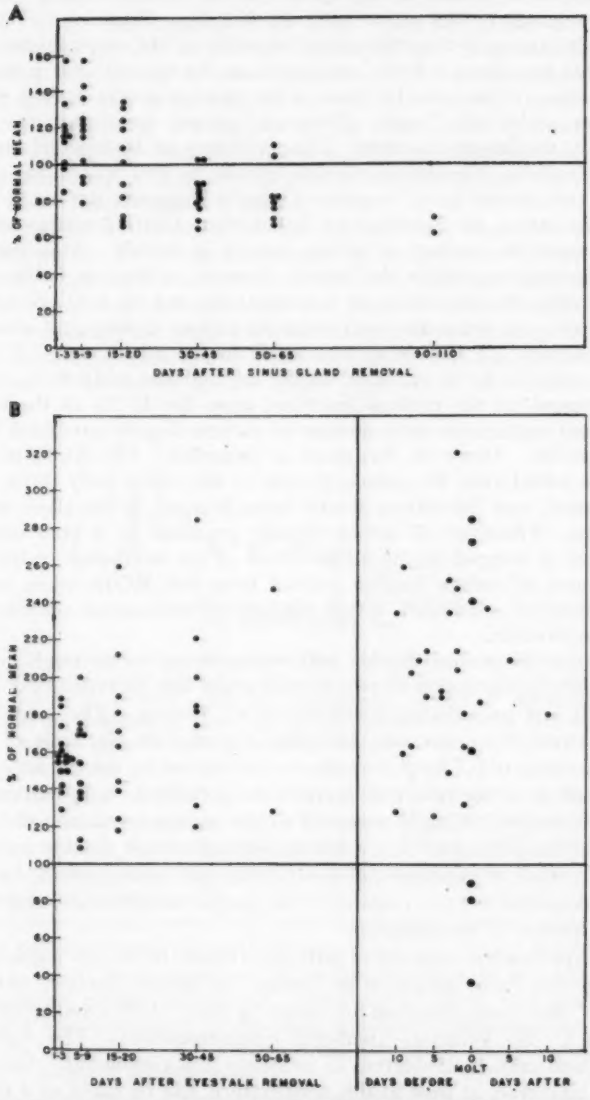

FIGURE 3. Rates of oxygen consumption of *Gecarcinus lateralis* after (A) bilateral removal of sinus glands and (B) bilateral eyestalk removal. Each value is plotted as per cent of the normal mean for that animal.

Observations

Contrasts in Metabolism Between Sinusglandless and Eyestalkless Crabs

1. *Respiratory rate*

Rate of oxygen consumption was determined as mm.^{3} per gram live weight per hour under standard conditions. Since it has been shown for the fiddler crab by Edwards (1950) that the absolute rate varies with sex and size, runs were limited to male animals, and all graphs intended to compare rates of many different animals have been expressed in per cent of each animal's normal average, as determined in two to four control runs.

Figures 3A and 3B indicate the striking differences between the rates of oxygen consumption of crabs from which only sinus glands have been removed and the respiratory rates of animals from which entire eyestalks have been taken. Figure 3A shows that after bilateral sinus gland removal, the rates of oxygen consumption tend in some animals to be high for the first few days but to be normal again within ten days to two weeks. The respiratory rates continue to drop, so that by the end of the first month they are below normal.

Bilateral eyestalk removal, on the other hand, causes the rates of oxygen consumption of all crabs to rise abruptly so that by the next day they are from 130% to 190% of normal. This rise may be followed by a temporary minor drop and then there is a gradual increase up to molt. On the day of molting, the respiratory rates have been variously recorded as 284% down to 36% of normal, but it is significant that the rates of those crabs which survived their molt were 284%, 273% and 240% of normal, whereas rates falling in the lower percentage range were recorded on animals which died during molt. It may be concluded that the respiratory rates are maximal at the time of molt. These observations on eyestalkless land crabs agree with those of Scudamore (1947) on crayfish and of Edwards (1950) on fiddler crabs. Scudamore, using the technique of Brown (1942), found that removal of sinus glands from three crayfish caused an increase in rates of oxygen consumption from a quarter to a fifth as great as the increase following eyestalk removal. Since his observations were limited to the first four days after surgery, he was probably observing in *Cambarus* a rise in respiratory rate comparable to that which the present writer has found may or may not occur in *Gecarcinus* following sinus gland removal and which, in any case, has disappeared within the ten days to two weeks following the operation.

2. *Respiratory quotient*

Each respiratory quotient was calculated as the total amount of carbon dioxide produced divided by the total amount of oxygen consumed during an experimental run.

The respiratory quotients of the entire series of normal animals tested from the beginning of November, 1950, until the end of June, 1951, are plotted in Figure 4. Each circle represents an individual determination and two to four separate circles represent the respiratory quotients for one animal. Therefore the one hundred and forty-odd circles represent the respiratory quotients of about forty animals. There is considerable variation in the normal respiratory quotient, which may range as high as 0.97 or as low as 0.61. The standard deviation of

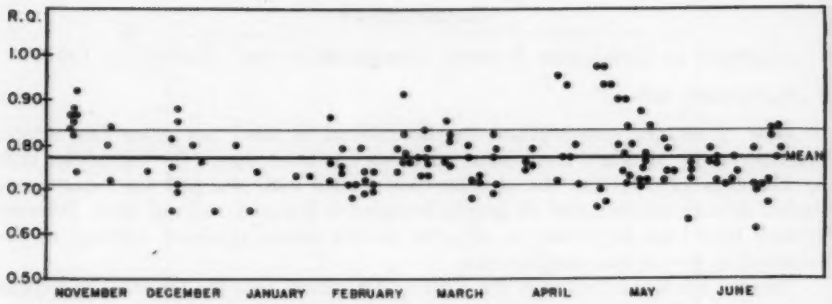

FIGURE 4. Respiratory quotients recorded from normal *Gecarcinus lateralis* from November, 1950, through June, 1951. The heavy line represents the mean respiratory quotient and the two narrow lines on either side of the mean indicate the range of plus and minus one standard deviation.

the mean respiratory quotients of 37 crabs is ± 0.06. The mean value is 0.77 ± 0.01.

In Figure 5A are shown the respiratory quotients for crabs from which both sinus glands have been removed. Each circle on the graph represents an individual determination but in this case the values are grouped, so that all determinations made between the first and the third day after sinus gland removal are in one column, those made between the fifth and ninth days in another, and so on. These values have an essentially normal distribution although there is a tendency, probably not of statistical significance (Table I), for a drop in respiratory quotient

TABLE I

The respiratory quotients of Gecarcinus lateralis

	Number in sample	Arithmetic mean and standard error	Probability (P)*
Normal Crabs	37	0.77 ± 0.01	
Eyestalkless Crabs			
1–3 days after operation	7	0.63 ± 0.02	<0.001
5–9 days after operation	6	0.69 ± 0.01	0.001
7–20 days pre-molt	16	0.68 ± 0.01	<0.001
Molting	7	1.40 ± 0.14	<0.001
Sinusglandless Crabs			
1–3 days after operation	10	0.72 ± 0.02	0.02
5–9 days after operation	11	0.74 ± 0.02	0.13
15–20 days after operation	11	0.79 ± 0.02	0.43
30–45 days after operation	11	0.77 ± 0.02	0.93
50–65 days after operation	8	0.75 ± 0.03	0.28

* P values are from table of t as given in Fisher and Yates (1948). A difference in mean respiratory quotient between normal and operated crabs is considered highly significant if P is less than 0.01.

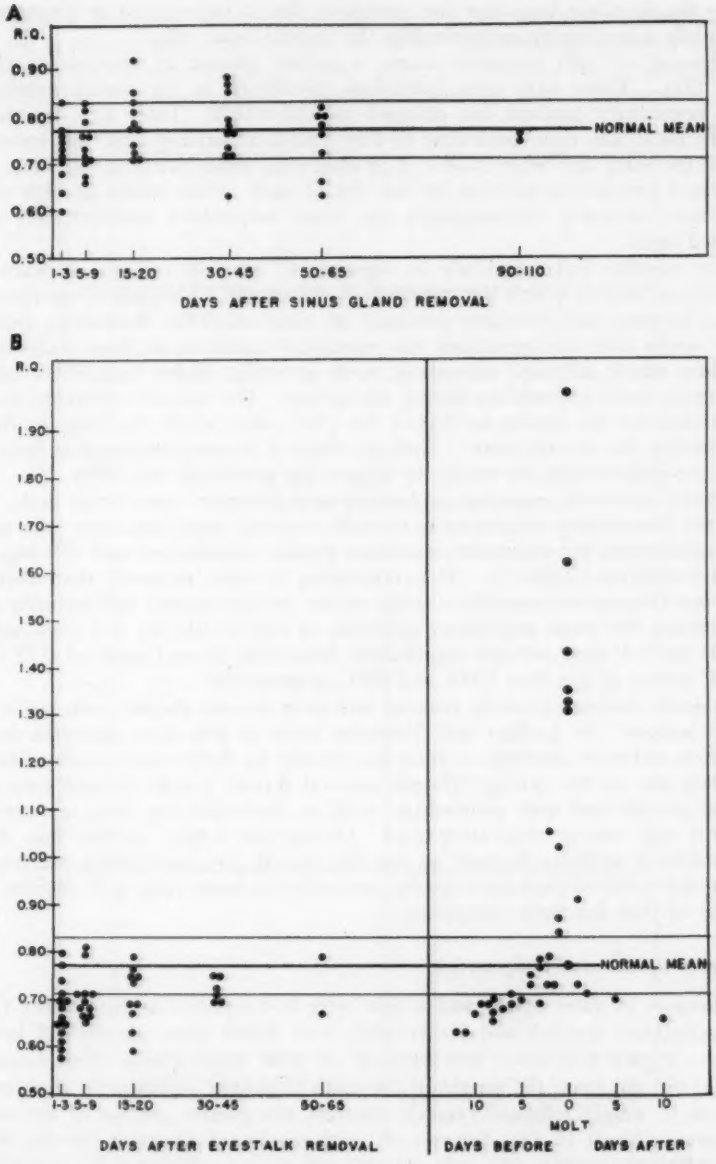

FIGURE 5. Respiratory quotients of *Gecarcinus lateralis* after (A) bilateral removal of sinus glands and (B) bilateral eyestalk removal. The normal mean and standard deviation are indicated as in Figure 4.

during the first few days after the operation, then a recovery to or above normal, and finally a leveling at approximately the normal mean value.

Removal of both eyestalks causes a radical change in respiratory quotient (Fig. 5B). Three days after operations performed in the spring months, the mean respiratory quotient has dropped to 0.63 ± 0.02 (Table I). By the fifth day the mean has risen somewhat to 0.69 ± 0.01, remaining near this value until around the tenth day before molt. A gradual rise, which starts at this time, culminates in a precipitous increase on the day of molt, when values as high as 1.97 have been recorded. Subsequently the mean respiratory quotient falls to the pre-molt level.

The rapidity and magnitude of response to eyestalk removal depends upon the time of year at which the operation is performed. Operations on four crabs during January and February produced no clear effect on respiratory quotients. Three weeks after the operations, the respiratory quotients of these crabs dropped to values which, although subnormal, were generally higher than those recorded from crabs made eyestalkless during the spring. The January-February data are responsible for the circles in Figure 5B (left side) which lie close to the line representing the normal mean. Similarly rates of oxygen consumption from these crabs are indicated by the circles in Figure 3B nearest to the 100% line. Since respiratory quotients recorded in January and February were from crabs which were not immediately responsive to eyestalk removal, these data have been omitted from calculations for respiratory quotients typical of crabs 1–3 and 5–9 days after eyestalk removal (Table I). It is interesting to note, however, that when data from non-responsive eyestalkless crabs of the winter months are included in the calculations, the mean respiratory quotients of 0.67 ± 0.02 for 1–3 days and 0.71 ± 0.01 for 5–9 days are still significantly below the normal mean of 0.77 ± 0.01, with P values of less than 0.001 and 0.01, respectively.

Intervals between eyestalk removal and molt become shorter with the advance of the seasons. In January and February forty to fifty days intervene between operation and molt, whereas in June only twenty to thirty days elapse. One may postulate that in the spring, eyestalk removal throws a crab at once into a full-fledged growth and molt metabolism, with an accompanying drop in respiratory quotient and rise in respiratory rate. During the winter months this shift in metabolism is partially blocked, so that the growth processes which are triggered by eyestalk removal start more slowly, proceed at reduced rates, and require longer periods of time for their completion.

3. *Water uptake and body weight*

Increases in water uptake and weight were first reported by Scudamore (1947) for eyestalkless crayfish and for crayfish from which sinus glands had been removed. Figure 6A shows that removal of both sinus glands from *Gecarcinus lateralis* did not cause the weight of the crabs to deviate significantly from normal. Increase in weight followed eyestalk removal, the greater portion of the increase commencing about 10 days before molt, with maximum values on the day of molt (Fig. 6B).

Responsible for the pre-molt weight increase in eyestalkless crabs was their

water uptake, becoming so marked as molt approached that the membrane linking abdomen to thorax bulged as if about to burst. The weights recorded on sinus-glandless crabs remained approximately unchanged because no increase in water uptake occurred. Intersegmental membranes were normal in appearance.

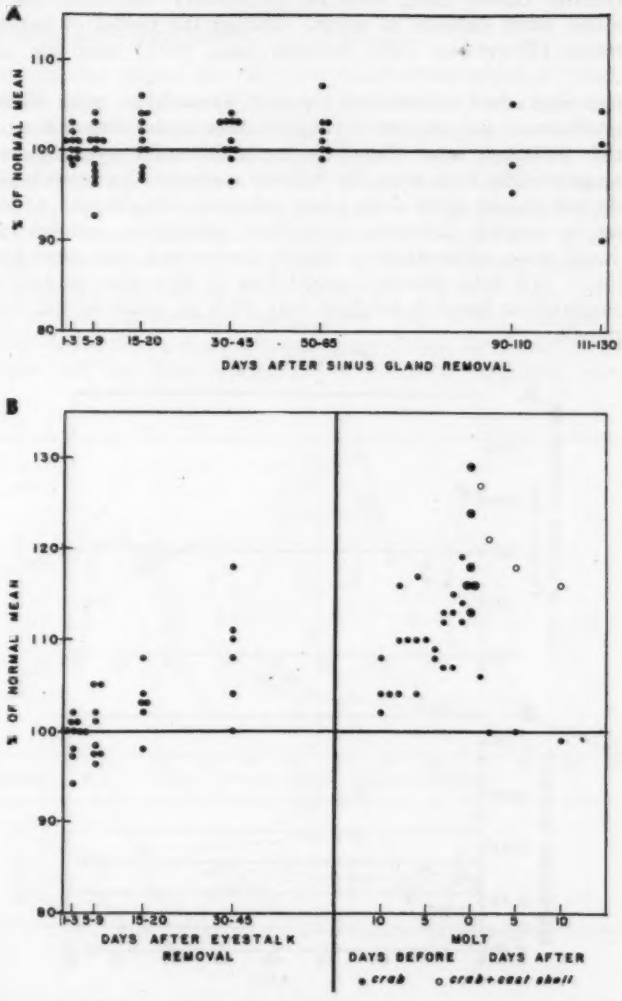

FIGURE 6. Wet weights of *Gecarcinus lateralis* after (A) bilateral removal of sinus glands and (B) bilateral eyestalk removal. Each value is plotted as per cent of the normal mean for that animal.

4. *Molt and gastrolith formation*

From the observations summarized in the preceding paragraphs it is apparent that, along with the relatively normal respiratory rate, respiratory quotient and water uptake which characterize sinusglandless crabs, there is no induction of molt. Eyestalk removal causes molt, with its preliminary alterations in respiratory metabolism and water balance, to occur. During the period of experimentation and observation (November, 1950, through June, 1951) only one normal crab molted.

Coincident with other preparations for molt, eyestalkless crabs remove calcium from the exoskeleton and deposit it as gastroliths under the chitinous lining of the stomach. Although from about the tenth day after eyestalk removal four pearly-white gastroliths form upon the calcium carbonate framework of the stomach, they do not appear after sinus gland removal. Scudamore (1947, p. 192) reported that in crayfish "bilateral sinus-gland extirpation resulted in gastrolith formation in all cases, although at a slightly slower rate than after bilateral eyestalk ablation." His data, however, reveal that 17 days after surgery the gastroliths of sinusglandless crayfish weighed only 23% as much as did those of eyestalkless animals.

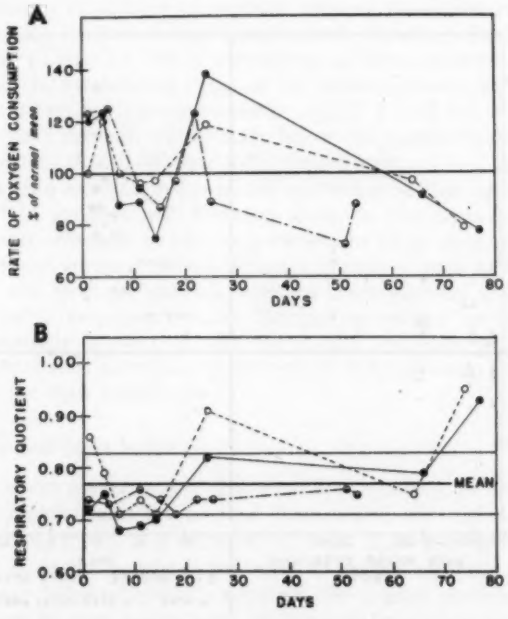

FIGURE 7. (A) Rates of oxygen consumption and (B) respiratory quotients of three normal crabs recorded over periods up to 77 days. The respiratory rates are indicated as per cent of each animal's mean. The normal mean respiratory quotient and standard deviation are indicated as in Figure 4.

The Respiratory Metabolism of Individual Crabs

The general pattern of response that is visible in group data presented in the previous section is apparent when the respiratory metabolism of individual crabs is followed for long periods.

1. *Normal respiratory metabolism*

In Figure 7A are plotted the rates of oxygen consumption of three crabs over a period of 77 days. Figure 7B shows the corresponding respiratory quotients during the same respiratory runs. Visible in both graphs is considerable fluctuation, indicating that a normal crab varies both its rate and type of metabolism. From a mean respiratory quotient of 0.77, it can be tentatively assumed that the foods principally oxidized are proteins and fats. Oil globules are visible in great quantities in the hepatopancreas. Fluctuations of respiratory quotient suggest, however, that conversion of carbohydrate to fat, producing higher respiratory quotients, and conversion of fat to carbohydrate, yielding lower values, may be involved in normal maintenance metabolism. The lower, steadier respiratory quotients recorded after eyestalk removal suggest that the fluctuating metabolism of the normal crab has been replaced by a relatively invariable metabolism, in

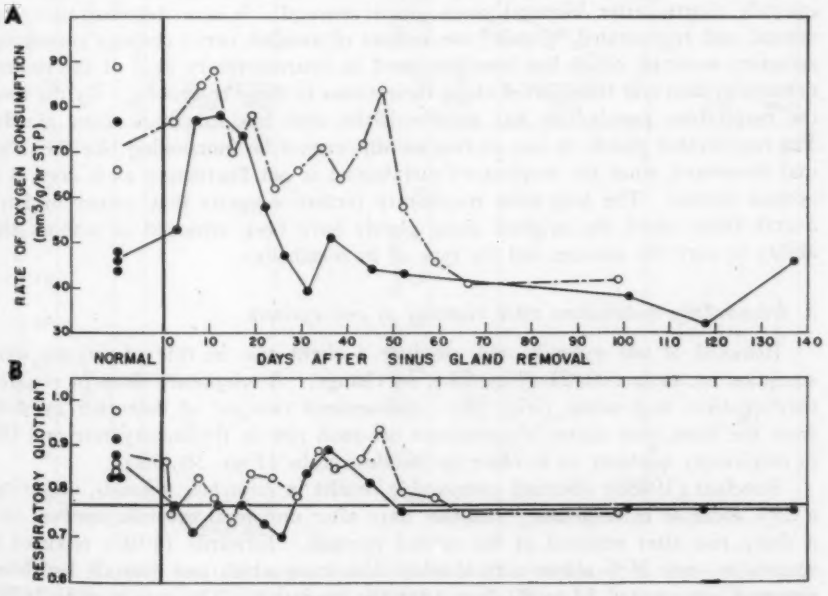

FIGURE 8. (A) Rates of oxygen consumption and (B) respiratory quotients of two crabs when normal and after bilateral removal of sinus glands. Open circles: crab 2; closed circles: crab 4. Solid horizontal line in (B): normal mean respiratory quotient.

which oxidation of fats and conversion of fats into organic acids, carbohydrates and other raw materials for the synthesis essential to growth play important roles.

2. *Respiratory metabolism after bilateral sinus gland removal*

One to two months after bilateral sinus gland removal there occurred stabilization of the respiratory quotient (Fig. 8B) close to the normal mean of 0.77 and of the respiratory rate (Fig. 8A) at normal or sub-normal levels. In six out of eight cases, of which one (crab 4) is shown in Figure 8A and another (crab 26) is illustrated in Figure 9C, the respiratory rate ascended immediately after the operation and then declined almost without fluctuations to reach, within the first month, a steady normal or sub-normal level. In the remaining two cases, of which one is illustrated in Figure 8A (crab 2), the respiratory rate subsequent to sinus gland removal varied near this crab's normal mean before stabilizing at a steady low level.

In four out of eight sinusglandless crabs, including crabs 2 and 4 of Figure 8B, the respiratory quotient fluctuated around the normal mean before stabilizing there, but in the remaining four individuals, of which one (crab 26) is shown in Figure 9D, the respiratory quotient fell sharply immediately after sinus gland removal and within three weeks had climbed smoothly to normal, where it stabilized.

In a previous paper (Bliss and Welsh, 1952) it was reported that a sinus gland starts to regenerate in an atypical position on the medulla terminalis of each eyestalk shortly after bilateral sinus gland removal. It was demonstrated that normal and regenerated "glands" are masses of swollen nerve endings containing secretory material which has been produced in neurosecretory cells of the central nervous system and transported along their axons to these reservoirs. By the time the respiratory metabolism has stabilized, the crab has substitute sinus glands. Yet regenerated glands of one or two months cannot be functioning like the original structures, since the respiratory metabolism is not fluctuating as it does in a normal animal. The long-term respiratory picture suggests that sooner or later a crab from which the original sinus glands have been removed is without the ability to vary the amount and the type of its metabolism.

3. *Respiratory metabolism after removal of one eyestalk*

Removal of one eyestalk may produce a slight rise in rate of oxygen consumption or, as in crab 21 (Fig. 9A), no change. A temporary drop in respiratory quotient may occur (Fig. 9B). Subsequent removal of the other eyestalk from the same crab causes a permanent pre-molt rise in respiratory rate and fall in respiratory quotient, as in other eyestalkless crabs (Figs. 3B, 5B).

Bauchau (1948b) obtained comparable results in *Eriocheir sinensis,* observing a 25% increase in respiratory rate five days after unilateral eyestalk removal and a sharp rise after removal of the second eyestalk. Edwards (1950) reported a respiratory rate 36% above normal when *Uca* from which one eyestalk had been removed were tested 14 to 39 days after the operation. The rate rose to 162% of normal when the second eyestalk was ablated.

The small rise observed by Bauchau after unilateral eyestalk removal was at-

tributed by him to insufficiency of secretion from the remaining sinus gland. If this interpretation is correct, Edwards' results indicate that compensation for the shortage of hormone by release of gradually increasing amounts from the intact gland does not occur. The slight intensification of respiratory metabolism correlates with the slight acceleration of molt frequency observed by Brown and Cunningham (1939) in crayfish possessing only one eyestalk.

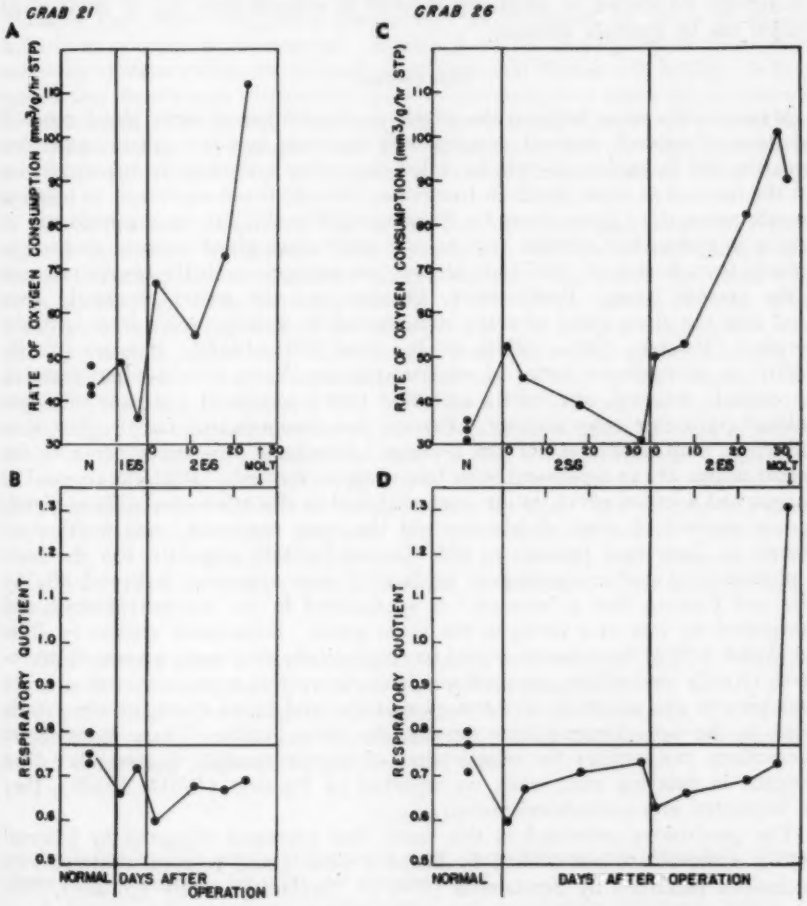

FIGURE 9. (A) Rates of oxygen consumption and (B) respiratory quotients of crab 21 when normal (N), after removal of one eyestalk (1ES) and after removal of the second eyestalk (2ES). The respiratory rates (C) and respiratory quotients (D) of crab 26 are illustrated when the crab is normal (N), without its sinus glands (2SG), and later without its eyestalks (2ES). Solid horizontal line in (B) and (D): normal mean respiratory quotient.

4. *Respiratory metabolism when bilateral sinus gland removal is followed by bilateral eyestalk removal*

The usual responses to eyestalk removal occur just as if there had been no previous surgical treatment, when bilateral eyestalk removal follows some time after bilateral sinus gland removal (Figs. 9C, 9D). These responses, recorded in three crabs, indicate that (1) the ability to respond to eyestalk removal is not altered by previous sinus gland removal, and (2) the molt-inhibitory hormone is permanently withdrawn or effectively reduced in concentration not by sinus gland removal but by eyestalk removal.

Discussion

Marked differences between the effects on respiration of sinus gland removal and those of eyestalk removal, coupled with contrasts in water uptake, gastrolith formation and induction of molt, have led the writer inevitably to the conclusion that the removal of sinus glands in *Gecarcinus lateralis* is not equivalent to bilateral eyestalk removal. Observations by Passano (1951b, 1952b) on the incidence of molting in crabs after eyestalk removal and after sinus gland removal (technique modified from Kleinholz, 1947) are in complete harmony with the results reported in the present paper. Furthermore, Passano and the writer separately have found that the sinus gland of crabs is connected by a large nerve tract with the x-organ [3] (Passano, 1951a, 1951b, 1952b; Bliss, 1951, 1952). Passano (1951b, 1952b), in an extensive series of experiments has shown that x-organ removal, like eyestalk removal, can induce molt and that implants of x-organs into eyestalkless crabs can delay molting. Passano has demonstrated for the first time a function, molt inhibition, for the x-organ. Results of two experiments by the present writer are in agreement with this work of Passano. Bilateral removal of x-organ and x-organ nerve, in one case with and in the other case without simultaneous removal of sinus glands, brought the same respiratory and molting responses in *Gecarcinus lateralis* as did bilateral eyestalk removal. On the basis of physiological and morphological evidence it was suggested independently by Bliss and Passano that a hormone [4] is synthesized in the x-organ of crabs and transported by way of a nerve to the sinus gland. Subsequent studies by Bliss and Welsh (1952) have demonstrated morphologically that many groups of neurosecretory cells situated in ganglia throughout the central nervous system of crabs participate in the synthesis of secretory material and in its transport along their axons to the two storage-release centers, the sinus glands. These more recent observations can explain the effectiveness of certain eyestalk tissues other than x-organs in delaying molt when, as reported by Passano (1951b, 1952b), they are implanted into eyestalkless crabs.

The conclusions presented in this paper that processes triggered by bilateral eyestalk removal are not evoked by bilateral sinus gland removal contrast with conclusions published by Scudamore (1947). Scudamore wrote (p. 205), "Bi-

[3] Probably the same discovery as was made in *Sesarma* by Enami (1951) who described these neurosecretory cells as "beta cells."

[4] Other than to suggest that the molt-inhibiting and respiration-regulating principles may be identical, the present studies have led to no conclusions concerning the number, identity and interrelationships of hormones being released by the sinus gland.

lateral eyestalk or sinus-gland excision resulted in gastrolith formation, removal of soluble salts from the exoskeleton, increase in water content, increase in oxygen consumption, and molting. . . ." *Cambarus,* used by Scudamore, responded to sinus gland removal (at 20% to 40% of the eyestalkless crayfish response with, however, only one molt) whereas, with the exception of a temporary change in mean respiratory rate and quotient, *Gecarcinus,* used by the present writer, did not respond in a molt-preparatory manner. In the light of recent physiological and morphological observations on crayfish, summarized in the next paragraph, it is probable that these contrasting results may be explained in terms of differing techniques of sinus gland removal. Proximity of the sinus gland to many neurosecretory centers within the eyestalk (see Bliss and Welsh, 1952, Figs. 1–3) requires that destruction of eyestalk tissue either during or following an operation be minimized. The method of sinus gland removal described in the present paper has the advantage, in common with the technique devised by Kleinholz (1947), of facilitating removal of a sinus gland with little derangement of other eyestalk tissues.

There are now indications that in crayfish the sinus glands can be removed without permanent interference with metabolism. Normal blood calcium levels were maintained and no incidence of molting occurred in crayfish from which sinus glands had been removed (Havel, 1949; Havel and Kleinholz, 1951). Sinus glandless crayfish were found to have a rate of oxygen consumption which was only slightly higher than that recorded after mock sinus gland operation and considerably below that of eyestalkless animals (Frost, 1948; Saloum, 1951; Frost, Saloum and Kleinholz, 1951). Mr. James B. Durand, Jr. (personal communication) has observed that nerve fibers from the x-organ and brain of crayfish carry stainable secretory material to their swollen endings, the sinus glands. In Macrura as in Brachyura there appears to be a neurosecretory system in which the sinus glands play the role of storage-release centers. The concept of a neurosecretory system, developed originally for vertebrates and insects (Scharrer and Scharrer, 1944; Bargmann and Scharrer, 1951; Scharrer, 1951; Scharrer, 1952a, 1952b) has now been extended to include the decapod Crustacea (Bliss and Welsh, 1952).

The writer wishes to express sincere thanks to Professor John H. Welsh for his constant encouragement, criticism and advice throughout the course of this study. Grateful acknowledgment is made to Dr. P. F. Scholander, Dr. Howard A. Schneiderman, Dr. Conrad Yocum and Dr. Richard Diamond for invaluable advice concerning respirometer practice and theory.

Summary

1. A volumetric macrorespirometer and gas analyzer are described. Procedures and precautions for their use are discussed.

2. Determinations of respiratory rates and respiratory quotients before and after surgical removal of both sinus glands from the eyestalks of the land crab, *Gecarcinus lateralis,* indicated that, except in one respect, the crabs were fundamentally unaffected by the loss of organs which had been thought essential for maintenance of normal metabolism.

3. The exception lay in an operated crab's eventual loss of its normal ability to vary the type and rate of metabolism. Indications of this loss were a constant respiratory quotient at the normal mean value and a low and relatively invariable respiratory rate. Fluctuations in rate of oxygen consumption and level of respiratory quotient were recorded from normal crabs.

4. Eyestalkless crabs showed a sudden and pronounced alteration from normal respiratory rates and respiratory quotients, a change indicative of a pre-molt metabolism and culminating in a second, even more marked metabolic shift at the time of molt.

5. It is clear that removal of eyestalks does, but removal of sinus glands does not, deprive these crabs of the molt-inhibiting and respiration-regulating hormone. Previous bilateral sinus gland removal does not alter the capacity of a crab to respond to subsequent bilateral eyestalk removal in the manner described above. The same is true when unilateral eyestalk removal is followed by removal of the other eyestalk.

6. These metabolic data supplement and confirm morphological observations reported in an earlier paper (Bliss and Welsh, 1952). One may conceive of a brachyuran neurosecretory system composed of neurosecretory cell bodies as synthesizing elements, axons as transporting elements, and the sinus glands as storing and releasing organs. This is an extension of a concept developed originally by Scharrer and Scharrer (1944) for vertebrates and insects.

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VARIATIONS IN CELL SIZE DURING THE DEVELOPMENT OF THE SLIME MOLD, DICTYOSTELIUM DISCOIDEUM [1]

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In many organisms, especially multicellular ones, it is relatively difficult to obtain an estimate of individual cell sizes, but in the amoeboid slime mold *Dictyostelium discoideum* it is not only possible to do this with reasonable accuracy for one stage, but for most of the stages of development. In the life cycle of this slime mold (Raper, 1935, 1951; Bonner, 1944) the amoebae are first separate during their actively feeding stage, and later after a short period of fasting they aggregate to form sausage-shaped cell masses which migrate for variable periods of time. During this migration stage the anterior cells begin their differentiation into stalk cells and the posterior cells begin their differentiation into spores (Bonner, 1952) and then finally the migrating mass shoots up into the air on a delicate stalk made up of the anterior cells which now have become vacuolated and encased in a hard cellulose sheath, and at the tip of this stalk there is a globular spore mass made up of cells encased in elliptical spore capsules. The range of individual cell sizes of each of these stages (with the exception of the mature stalk cells which could not be measured accurately because of their irregular shape) was determined and it was found that changes in the stage of development as well as early signs of differentiation were all reflected in characteristic changes of individual cell sizes.

Materials and Methods

The method of culture and the culture medium were the same as those used previously and the aggregating and migrating amoebae were prepared by centrifuging the vegetative amoebae free of bacteria and placing them on plain agar (Bonner, 1947). The cells at each stage were removed and placed in a drop of standard solution (NaCl, 0.60 gm.; KCl, 0.75 gm.; $CaCl_2$, 0.30 gm.; distilled H_2O, 1000 ml.) on a microscope slide. A No. 1 coverslip (22 × 22 mm.) was placed over the drop, but supported on two bits of coverslip so as to prevent the cells from being crushed. The diameters of the rounded, spherical cells were then measured with a 95 X, oil immersion objective and a Zeiss filar micrometer. In the case of the spores, which are elliptical in shape, both the long and the short axes were measured, and the spore diameter is expressed as the mean of these two values for each spore measured.

A number of tests were run to determine if the diameter of an individual cell remained constant when immersed in standard solution. It was found that during a period from 5 to 90 minutes after immersion the cell diameter did remain fairly constant, the small fluctuations being apparently caused by the activity of the contractile vacuole.

[1] This work was carried out with the help of a grant from the American Cancer Society and with funds of the Eugene Higgins Trust allocated to Princeton University.

The accuracy is considered to be most reasonable for the vegetative amoebae and the spores. However, during the aggregation and migration stages a fair per cent of the cells either did not round up after being subjected to the salt solution, or they remained part of a mass of cells and were impossible to measure. Therefore, during these two stages, there might conceivably be a sizable error due to having selected only those cells that were spherical. In defense of this it can only be said that there was no apparent or striking size difference and that it is believed that if an error has been introduced by this procedure it is likely to be relatively small.

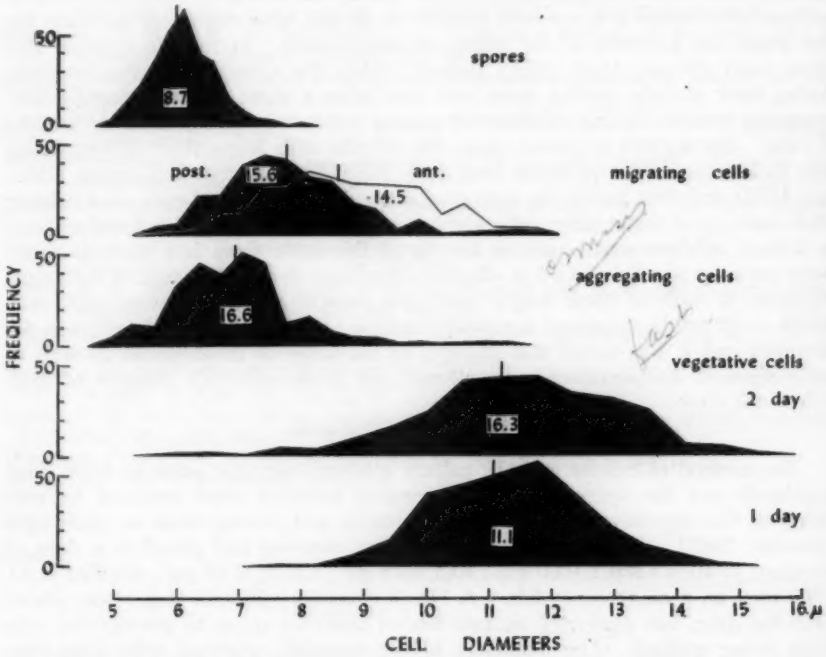

FIGURE 1. A graph in which the frequency is plotted against the cell diameter for various stages of development. Each curve consists of 300 observations. The vertical lines indicate the mean values. The numbers placed within the curves are the coefficients of variation in per cent.

RESULTS

The diameters of 300 cells were measured for the following stages of development: (1) vegetative amoebae approximately one day old (between 22.5 and 23.5 hrs.), (2) vegetative amoebae approximately two days old (between 41.5 and 46 hrs.), (3) aggregating amoebae, (4) and (5) the anterior and posterior cells at the beginning of the migration stage (3 to 4 mm. of migration) and (6) the mature spores. For each stage, this count of 300 was made on three separate

samples of 100, except in the case of the migrating cells which were measured in four samples (anterior) and five samples (posterior).

An analysis was made of the measurements at each of these stages to determine which of the three quantities, diameter, surface area or volume, would give a more normal distribution when plotted against frequency. It was found, by plotting the cumulative relative frequency against these three quantities on probability paper, that for all of the stages of development measured, the diameters gave the curve with the least skewness.[2] In fact the two vegetative amoebae curves as well as the anterior migrating cells and the spores very closely approximated the normal distribution curves, while the aggregating cells and the posterior migrating cells showed a certain skewness in the higher end of their range.

If the frequencies are plotted against diameters for all the different stages and are shown on one graph, as has been done in Figure 1, it becomes obvious that the mean size (indicated by the vertical lines) goes through considerable fluctuations during the course of development. It remains constant throughout the vegetative period but makes a dramatic drop during the aggregation stage. During the migration period, the cells increase in size, but most interesting here is the fact that the anterior presumptive stalk cells are highly significantly larger than the posterior presumptive spore cells. A spot check was made on 50 anterior and 50 posterior cells of an old migrating cell mass (one that had migrated 25 mm.) and it was found to be the same as the young cell masses tested. The spores, on the other hand, are the smallest of all the stages.

In glancing at Figure 1, one can see that the range varies for each stage and an analysis was made to see to what extent this was related to size, for the larger the mean, the larger would be the expected range if the amount of variation about the mean were the same. To compare the different stages the usual procedure of dividing the standard deviation by the mean was employed to obtain the coefficient of variation. These coefficients are indicated on Figure 1 and it can be seen that during the first day of vegetative growth the variability is low but that it rises during the second day and remains high in the migration stage, only to drop to its lowest value in mature spores.

Discussion

It is not surprising to find that the mean size does not change between one and two days of vegetative growth, and the cause of the sudden drop during aggregation is perhaps understandable for the vegetative amoebae are actively feeding and engulfing bacteria, while aggregating cells have been fasting for a considerable period of time. (In this case it has been some 17 to 18 hours since the amoebae were washed free of most of the bacteria by centrifugation.) There is no known explanation why the cells increase in size during the migration stage, but certainly it cannot involve feeding but must involve internal osmotic changes. The most important fact is that in the migrating cell mass the anterior presumptive stalk cells are significantly larger than the posterior presumptive spore cells, giving another example of an early detectable difference in differentiation. These migrating cell masses were stained with vital Nile blue sulfate and show characteristic dark anterior ends and light posterior ends (Bonner, 1952).

[2] The authors are greatly indebted to Dr. M. H. Belz, visiting Professor at the Department of Mathematics, Princeton University, for his help in the statistical work in this paper.

The variability during certain stages is remarkably high in *Dictyostelium* when one compares it to the variability of various unicellular organisms listed by Adolph (1931). To fully appreciate the consequences of this, one need only visualize the volumes; the largest two-day old vegetative amoebae, for instance, may be 18 to 19 times greater in volume than the smallest ones.

In comparing the coefficients of variation of the different stages there is an interesting trend, for the variability is low during the spore stage and rises somewhat after one day of vegetative existence, but only reaches a peak of variability after two days.

It is a curious fact that for these cell populations the diameters should give a normal distribution curve rather than the surface areas or volumes. Of course, since the factors which determine size, variability and skewness are virtually unknown, there is no reason to expect any particular type of distribution curve. An intriguing possibility might be, however, that the linear dimension gives a normal distribution because a ratio of the volume to the surface is in some way limiting to cell size and this ratio is, of course, linear. But unfortunately there is at the moment no way to weigh down such a wild speculation with a few facts.

Summary

1. The individual cell size was measured for 300 cells at various stages during the development of the slime mold *Dictyostelium discoideum;* during two periods of the vegetative stage when the amoebae are actively feeding, during the aggregation stage when the amoebae are streaming together to form cell masses, during the migration stage when the cell mass wanders over the substratum, and finally the mature encapsulated spores.

2. The mean size was large during the vegetative period, dropped severely in the fasting aggregating amoebae, increased slightly during the migration stage, only to fall to their minimum size as mature spores.

3. The variability in size was large and especially so during the periods before, during and after aggregation.

4. Of special interest relative to the problem of differentiation was the fact that during the migration stage, the anterior presumptive stalk cells were significantly larger than the posterior presumptive spore cells, being another example of an early detectable sign of differentiation.

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RELATIONS BETWEEN PRE- AND POST-ANAEROBIC OXYGEN CONSUMPTION AND OXYGEN TENSION IN SOME FRESH WATER SNAILS

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Aquatic snails cannot be considered as particularly well adapted to anaerobic life since, in the absence of oxygen, they eventually lose their motility and die. They can, however, tolerate anaerobiosis for longer or shorter periods, these periods varying with the external temperature and also with the species (Jatzenko, 1928; Alsterberg, 1930; von Brand, Baernstein and Mehlman, 1950). Furthermore, it has been shown that some of the end products resulting from the anaerobic carbohydrate breakdown accumulate in the tissues (Mehlman and von Brand, 1951).

Animals giving this general type of response to lack of oxygen usually show in a subsequent aerobic recovery period a temporarily increased rate of oxygen consumption, a phenomenon variously known as repayment of an oxygen debt, respiratory rebound, or respiratory overshoot. Very little is known about this phase of snail physiology, and the few data available are contradictory. Borden (1931) found a very marked respiratory rebound in *Planorbis corneus*, while Füsser and Krüger (1951) did not find in the post-anaerobic respiration of this species and that of *Lymnaea stagnalis* a significant increase over the pre-anaerobic level.

Particular attention is paid in the present paper to the question of the relation between oxygen tension and pre- and post-anaerobic rates of oxygen consumption. It has been shown in many other invertebrates that the post-anaerobic oxygen consumption is frequently more dependent on the tension than the pre-anaerobic one. It has been claimed on the one hand that this indicates the participation of a different enzyme mechanism during the recovery period (Harnisch, 1935, 1936, 1951), while on the other hand von Buddenbrock (1939) considers the phenomenon as a necessary consequence of the increased respiratory level which would change the critical point at which diffusion ceases to furnish the organisms all the required oxygen. It has been pointed out previously (von Brand, 1947) that an extensive investigation on some non-parasitic invertebrate would be necessary to clarify the situation.

Materials and Methods

The following species of snails were used:

Planorbidae: *Australorbis glabratus*, laboratory-reared from Venezuelan stock, and *Helisoma duryi*, collected at Kenilworth, Maryland, but maintained in labora-

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tory aquaria for at least a week before being used. The Planorbidae employed ranged in weight from about 150 to 350 mg.

Physidae: Laboratory-reared *Aplexa nitens* from Texan stock, weighing from 200 to 500 mg.

Lymnaeidae: *Lymnaea stagnalis*, laboratory-reared, colony derived from laboratory-reared specimens obtained from Dr. L. E. Noland, University of Wisconsin. Juveniles weighing from about 100 to 600 mg. were used.

All respiration experiments were done by means of the conventional Warburg technique; vessels of about 16 ml. capacity were used, and 2 ml. of dechlorinated tap-water served as medium. Each specimen was used only for one experiment. The determinations were done at 30, 20 and 10° C. In general, a single snail was placed in a Warburg flask, but at 10° C. two snails frequently were used in order to obtain manometric readings of sufficient magnitude.

The usual experimental procedure was as follows: The rate of oxygen consumption was first established for 2.5 to 4 hours under atmospheric air (21 per cent oxygen). The gas phase was then changed, if required, by flushing the manometers with 100 per cent oxygen, 11 or 5 per cent oxygen in nitrogen (gases taken from steel cylinders; composition verified by analysis) and the respiratory rate again determined during 3 to 4 hours. Anaerobiosis was then established by flushing the manometers for 15 to 25 minutes with pure Linde nitrogen (99.99 per cent, further purified by passing over heated copper, pressure difference in manometer ca. 100 mm. Brodie fluid), and anaerobic conditions were maintained for 16 ± 0.5 hours. Shaking of the manometers was suspended after anaerobiosis was established since during this period no readings were taken. At the end of the 16 hours the vessels were removed from the manometers, the medium was withdrawn by means of a Pasteur pipette and replaced by fresh medium. In this way the excreted end-products were eliminated. They could otherwise have been reabsorbed or served as substrate for bacterial development during the recovery period. The manometers were then returned to the water bath and flushed with the same gas which had been used immediately preceding the anaerobic period. This flushing period served at the same time as temperature re-equilibration period. Readings were then taken usually for 8 hours, but in a few cases only for 7 or 7.5 hours.

Both pre- and post-anaerobic readings were taken at 15-minute intervals, but in experiments with *Australorbis glabratus* and *Helisoma duryi* at 10° C. the interval was 30 minutes. The data for each reading interval of each experiment were calculated separately. They were averaged and graphed for a given series. Owing to the many readings available for each experimental period, the respiratory pattern could be established with considerable accuracy. The experimental procedure permitted the direct comparison of the post-anaerobic oxygen consumption with the pre-anaerobic rate in both 21 per cent oxygen and in the same gas that was used immediately preceding the anaerobic period. In a few experiments, the pre-anaerobic relation between oxygen consumption in 21 per cent oxygen and the other tensions was established separately. Where in such cases post-anaerobic determinations were done, different snails were used and only one of the four gas mixtures was used both pre- and post-anaerobically.

TABLE I

Influence of oxygen tension on the pre- and post-anaerobic oxygen consumption of well-fed snails

Species	Temp. °C.	No. of expts.	Pre-anaerobic rates			Post-anaerobic maximal rate	
			Rate in 21 per cent O_2 mm.$^3O_2$/gm./hr.	O_2 per cent in exp. gas	Rate in exp. gas in per cent of rate in 21 per cent O_2	In per cent of pre-anaerobic rate in 21 per cent O_2	In per cent of pre-anaerobic rate in exp. gas
Australorbis glabratus	30	17	119.0±6.4	100	109±5.0	170±6.3	157±7.0
Australorbis glabratus	30	16	120.9±5.2	21	100	162±10.3	162±10.3
Australorbis glabratus	30	14	148.7±8.8	11	104±5.7	147±7.8	141±7.5
Australorbis glabratus	30	31	144.8±6.4	5	66±4.6	89±3.7	135±5.6
Australorbis glabratus	20	12	50.2±2.9	100	114±6.4	151±9.9	133±5.0
Australorbis glabratus	20	18	69.2±2.8	21	100	122±4.7	122±4.7
Australorbis glabratus	20	12	56.0±6.0	11	86±9.6	142±13.3	173±13.1
Australorbis glabratus	20	11	62.7±5.0	5	69±6.8	80±7.7	122±13.9
Australorbis glabratus	10	20	18.8±0.6	100	98±3.9	148±6.2	154±9.8
Australorbis glabratus	10	18	11.1±0.4	21	100	130±7.4	130±7.4
Australorbis glabratus	10	22	18.6±1.1	11	77±2.4	134±6.7	178±11.2
Australorbis glabratus	10	20	17.4±0.6	5	66±3.9	107±7.2	170±18.3
*Australorbis glabratus**	20	14	52.0±3.7**	100	114**	189**	170±6.9
*Australorbis glabratus**	20	17	58.6±4.8	21	100	145±8.0	145±8.0
*Australorbis glabratus**	20	13	72.7±3.8**	11	86**	120**	143±10.7
*Australorbis glabratus**	20	21	59.3±2.4**	5	69**	94**	142±7.3
Helisoma duryi	30	15	125.9±13.2	100	111±6.2	211±13.6	192±12.4
Helisoma duryi	30	16	137.3±3.4	21	100	156±6.0	156±6.0
Helisoma duryi	30	14	185.9±10.5	11	81±4.1	138±7.2	170±8.9
Helisoma duryi	30	14	181.8±4.7	5	50±4.0	72±3.3	152±6.6
Helisoma duryi	20	14	56.2±3.6	100	93±4.3	141±9.5	154±9.5
Helisoma duryi	20	13	49.2±5.2	21	100	166±10.0	166±10.0
Helisoma duryi	20	13	49.3±3.4	11	83±3.0	132±12.4	157±8.3
Helisoma duryi	20	12	45.6±4.6	5	64±3.0	112±8.3	179±13.7
Helisoma duryi	10	16	15.5±1.0	100	87±6.9	197±16.0	232±16.6
Helisoma duryi	10	16	13.7±0.7	21	100	148±9.5	148±9.5
Helisoma duryi	10	16	13.2±1.0	11	93±5.9	138±13.0	155±13.4
Helisoma duryi	10	16	15.0±0.4	5	79±3.6	168±18.3	217±18.3
Aplexa nitens	20	12	50.3±4.2	100	126±6.6	175±13.3	139±9.7
Aplexa nitens	20	12	59.9±7.0	21	100	154±11.6	154±11.6
Aplexa nitens	20	16	62.6±1.7	11	85±7.5	133±4.5	156±5.7
Aplexa nitens	20	9	42.1±4.5	5	74±9.9	144±23.1	186±31.6
Lymnaea stagnalis	20	14	150±7.6	100	93±4.7	128±6.0	139±7.1
Lymnaea stagnalis	20	15	84.9±9.1	21	100	115±4.6	115±4.6
Lymnaea stagnalis	20	16	149.6±12.3	11	73±3.2	69±6.6	95±8.1
Lymnaea stagnalis	20	14	109.5±9.4	5	52±2.8	54±5.8	105±10.0
Lymnaea stagnalis	10	17	41.8±1.3	100	96±3.2	117±1.1	124±6.2
Lymnaea stagnalis	10	17	35.4±1.3	21	100	122±9.2	122±9.2
Lymnaea stagnalis	10	16	35.3±2.0	11	91±4.8	106±5.3	116±3.6
Lymnaea stagnalis	10	17	34.2±2.3	5	60±3.5	74±5.0	126±6.8

The oxygen determinations were done at the same temperature used for the anaerobic exposure in all series, except those marked with an asterisk. In these the oxygen determinations were carried out at 20° C., but the snails were exposed to anaerobiosis at 30° C. In this series only the experimental gas was used pre-anaerobically; the values marked by two asterisks are calculated on the assumption that the pre-anaerobic oxygen consumption showed the same dependency on the tension as shown by the corresponding series done with the same species at the same temperature and shown elsewhere in this table. All averages are arithmetic averages and hence show a slight upward bias. The figures after the ± signs are the standard error of the mean.

Results

1. Oxygen tension and pre-anaerobic oxygen consumption

A study of the pre-anaerobic rates of oxygen consumption of *Australorbis glabratus* (Tables I and II) shows that in this species the percentage decline in oxygen consumption at tensions below 21 per cent oxygen was on the whole remarkably similar regardless of whether the metabolic rate was changed by using different temperatures or snails of different nutritional state. One curious observation was that the oxygen consumption remained virtually unaffected by lowering the oxygen concentration from 21 to 11 per cent at 30° C., while the rate was definitely lowered under analogous conditions at 20° and 10° C. It is not believed that this somewhat paradoxical finding is to be attributed to experimental errors because in previous experiments (von Brand, Nolan and Mann, 1948) a similar, or even somewhat more pronounced independence of the oxygen consumption on the tension had been found at 30° C.

Table II

Influence of starvation on the dependency of the pre-anaerobic oxygen consumption on the oxygen tension. The rates are expressed in mm.³ oxygen per gm. fresh weight per hour

Species	Temp °C.	No. exp.	Starvation days	Oxygen consumption in per cent oxygen		5 per cent rate in per cent of 21 per cent rate
				21	5	
Australorbis glabratus	30	31	0	144.8±6.4	95.7±4.2	66±4.6
Australorbis glabratus	30	17	14	64.1±1.4	46.7±2.5	73±3.7
Australorbis glabratus	20	11	0	62.7±5.0	41.3±3.6	69±6.8
Australorbis glabratus	20	23	14	34.2±1.3	22.7±1.8	66±4.2
Helisoma duryi	30	14	0	181.8±4.5	90.9±4.7	50±4.0
Helisoma duryi	30	12	14	55.6±6.3	42,4±4.0	78±2.5
Helisoma duryi	20	12	0	45.6±4.6	27.9±2.3	64±3.0
Helisoma duryi	20	10	14	29.4±1.9	20.4±1.6	73±7.1

In *Helisoma duryi* and *Lymnaea stagnalis*, it is evident that lowering the metabolic rate did lead to a reduction in the dependency of the respiratory rate on the tension, although the effect was not very pronounced.

In view of these variations it is difficult, if not impossible, to grade the snail species used as to the magnitude of influence of oxygen tension. Apparently only minor differences exist in this respect between the four species investigated and, within the range of tensions tested, the species with hemoglobin in their blood (*Australorbis* and *Helisoma*) are, at a given temperature, capable of securing only a slightly higher percentage of their maximal oxygen needs than the snails having hemocyanin (*Lymnaea* and *Aplexa*). This is in approximate agreement with the findings of Füsser and Krüger (1951) on *Planorbis* and *Lymnaea*. We are also in agreement with these workers as to the finding that the actual respiratory rate, at equal temperature, is higher in *Lymnaea* than the other species. Evidently, the temperature relationships of various snail species must vary, since in previous experiments no such difference was found at 30° C. (von Brand, Nolan and Mann, 1948).

2. Post-anaerobic respiration

All four snail species studied showed a marked respiratory rebound after having been exposed to 16 hours of anaerobiosis (Table I). In all cases the period of excess oxygen consumption lasted a long time and three different types of recovery curves could be distinguished. In type 1, characteristic for *Helisoma*, the post-anaerobic rate was higher than the pre-anaerobic level immediately after restoration of aerobic conditions (excluding, of course, the time necessary for changing the medium and the other necessary manipulations, for which no data are available). The rate remained at a high level for several hours and then began to approach the normal without, however, reaching it completely within the time of observation. In type 2, occurring frequently in *Australorbis* and *Lymnaea*, the oxygen consumption was increased from the beginning and remained at this same level for the entire period of 7 to 8 hours. In type 3, observed in some series of all snails and the only one seen in *Aplexa*, the post-anaerobic rate was initially relatively low. After a period, varying from about one to three hours, a transition to maximal respiratory activity (higher than pre-anaerobic) occurred and this rate was usually sustained for the balance of the observation period. In a few cases, especially with *Australorbis*, a distinct lowering of the rate occurred during the 7th or 8th hour. During the initial period, the oxygen consumption was either somewhat lower or higher than the pre-anaerobic rate, but in most instances it was surprisingly close to the pre-anaerobic level, giving, perhaps erroneously, the impression that the normal (pre-anaerobic) metabolism was resumed immediately after restoration of aerobiosis, but that a certain induction period was necessary in these cases to initiate the actual respiratory rebound.

It is evident that owing to the long periods of "repayment" no definite data can be given concerning the total amount of oxygen repaid. However, in those cases where, after an initial high level, the post-anaerobic rate later approached the pre-anaerobic rate, it can be stated with some certainty, on the basis of a few observations made after 24 hours, that the repayment was far from complete. This is in contrast to the findings of Borden (1931) on *Planorbis corneus*. Borden used only very short periods of anaerobiosis; we used long periods. It is very probable that the proportion between anaerobic end-products accumulating in the tissues and being excreted varies under such different conditions and that this factor may be responsible for the differences in results. In our opinion the significance of the term "complete repayment of an oxygen debt" is sometimes over-emphasized. Insofar as invertebrates are concerned it does not imply anything of fundamental importance. The total amount of oxygen repaid will depend primarily upon the level of anaerobic metabolism, the nature of the anaerobic end-products, and the question to what extent they are excreted or stored in the tissues. None of these factors has necessarily a close quantitative connection with the pre-anaerobic oxidative metabolism, that is, with the amount of oxygen missed during anaerobiosis, but they will decide whether the post-anaerobic overshoot leads to an incomplete or complete repayment of the oxygen debt, or even to a more or less marked overpayment (review of the pertinent literature in von Brand, 1946).

Special attention was given to the relationship existing between pre-anaerobic rate of oxygen consumption and maximal post-anaerobic rate sustained over at

least two hours and usually over a much longer period. A study of Table I shows that in most series the post-anaerobic respiration was more dependent on the oxygen tension than the pre-anaerobic one if the pre-anaerobic consumption at 21 per cent oxygen is taken as reference point for both. It is also evident that

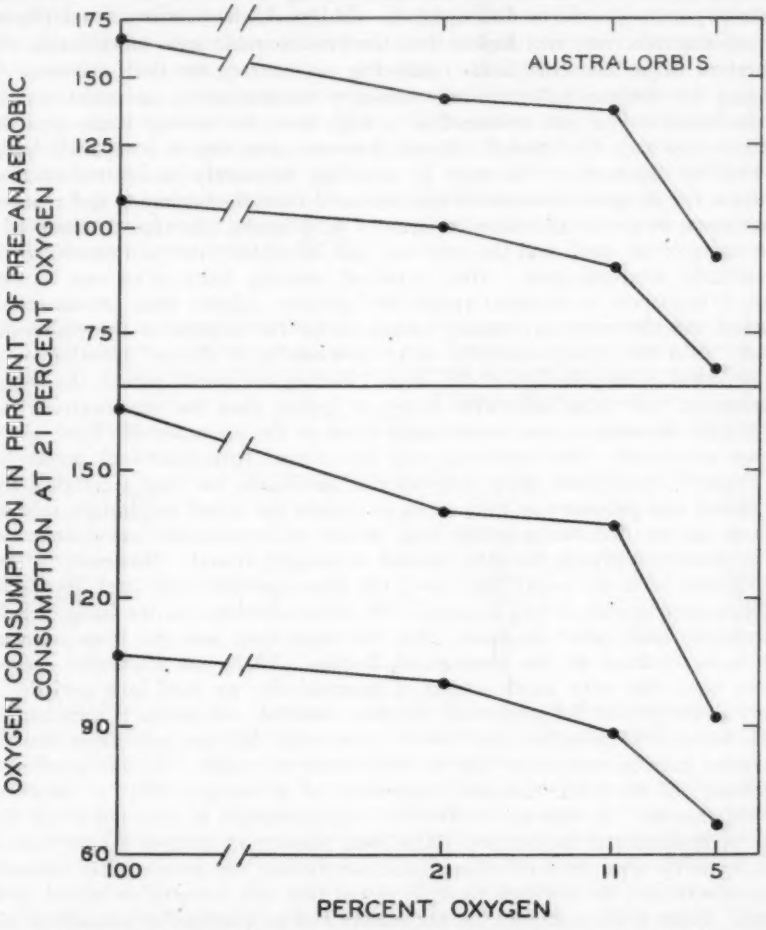

FIGURE 1. Dependency of the pre- and post-anaerobic respiration of *Australorbis glabratus* on the oxygen tension. Average values of all series plotted on an arithmetic scale (lower part of figure) and logarithmic scale (upper part of figure).

changes in the metabolic level induced by temperature changes have no decisive influence on the relation between pre- and post-anaerobic rates of oxygen consumption. The variations between the various series conducted with one and the same

snail species are fairly large, but this will surprise no one who has had experience with metabolic studies on this group of organisms.

Undoubtedly, some differences exist in the pre- to post-anaerobic ratios for the various conditions considered which appear significant from a purely statistical standpoint. By and large, however, the ratios are in the same region at all temperatures studied. Moreover, no constant pattern of temperature differences could be found when different oxygen tensions were used. Presumably, the significant differences reflect differences in the various batches of snails rather than differences due to temperature or oxygen tension. Separate batches of snails had to be used for each series and the entire experiments spread out over about a year.

As mentioned in a previous section the percentage decline in pre-anaerobic respiration at lowered oxygen tensions is unaffected by temperature changes only in *Australorbis*. It appears, therefore, permissible only in this species to average all the available series in order to minimize the biological variations by using the greatest possible number of observations. The results are shown in Figure 1. When plotted on an arithmetic scale, as has been done heretofore by all investigators, the greater dependency of the post-anaerobic oxygen consumption on the oxygen tension is quite evident. If considered from this standpoint alone, similar findings of Harnisch (1935, 1936) on insects and worms are apparently confirmed. If, however, the data are plotted on a logarithmic scale, a different picture emerges. The lines for the pre- and post-anaerobic respiration now rather closely parallel each other, indicating that the percentage increase of the post-anaerobic respiration over the pre-anaerobic rate is approximately identical at all tensions studied. The deviations of the two lines upon plotting on an arithmetic scale are quite apparently attributable to rate differences sustained by the pre-anaerobic respiration at the various oxygen tensions. Therefore, they actually do not constitute a proof for the contention that the post-anaerobic respiration is more dependent on the tension; rather, they prove the contrary.

The situation is essentially the same in the other snail species investigated. Again, considerable differences within series exist (Table I). The trend of the figure, however, is unmistakable. In all species, the post-anaerobic oxygen consumption is raised over the pre-anaerobic level to about the same degree regardless of the oxygen tension. A survey of Table I shows that *Helisoma* raised its post-anaerobic level highest, that of *Lymnaea* remained always relatively low, and the two other species were intermediate.

Discussion

The present investigation has shown that the oxygen consumption of four species of pulmonate snails is somewhat dependent on the tension below 21 per cent oxygen and that this dependency shows little connection with the actual rate of oxygen consumption. In *Australorbis glabratus* held in 5 per cent oxygen, the percentage decline (around 34 per cent) was the same whether (1) the actual respiratory level in 21 per cent oxygen was reduced from 145 $mm.^3$/gm./hr. to 17 $mm.^3$ by changing the temperature from 30° C. to 10° C., or (2) from 145 $mm.^3$ to 64 $mm.^3$ by starvation. In *Lymnaea stagnalis* and *Helisoma duryi*, the dependency was not quite as unalterable. In the latter species, for instance, the percentage decline in 5 per cent oxygen was 50 per cent when the metabolic level

was 182 mm.^{3}/gm./hr. in 21 per cent oxygen, 36 per cent at a level of 46 mm.^{3} and 21 per cent at a level of 15 mm.^{3}. It is evident that even in this latter case the oxygen consumption does not entirely conform to what would be expected if diffusion were the only limiting factor. According to the diffusion hypothesis, which at present seems to be accepted almost universally, lowering the oxygen tension below a critical point leads to a lowering of the over-all consumption because at lower tensions all the oxygen entering the body by diffusion is consumed by the superficial tissue layers and an internal layer becomes anoxic. Since temperature has but little influence on the rate of diffusion and starvation probably has none,[3] it would appear that the oxygen consumption of the above snails held in 5 per cent oxygen at 10° C. should have been about 100 per cent of that shown in air if the diffusion hypothesis held strictly. This seems to follow from the observation that at 30° C. in 5 per cent oxygen diffusion was able to bring much more oxygen into the body than the snails consumed at 10° C. in 21 per cent oxygen.

The above argument is not quite straightforward, however, because the snails have a circulatory system. It is conceivable that the oxygen transport within the body and/or the influence of temperature on the loading and unloading tension of the respiratory pigments may be the limiting factors. In order to shed some light on this point, several observations on the rate of heart-beat were done. In 15 *Australorbis* it was reduced at 10° C. to 13 per cent of the 30° rate, while the rate of oxygen consumption was reduced to 11 per cent. Similar experiments with 6 *Lymnaea* showed at 10° C. reductions in both rates corresponding to 44 and 43 per cent, respectively, of the 20° C. rate. This close parallelism between the rates of heart-beat and oxygen consumption tends therefore to support the above view. However, no such close parallelism was found in respect to the influence of starvation. A starvation period of two weeks reduced the rates of heart-beat and oxygen consumption of 15 *Australorbis* to 75 and 44 per cent of the pre-starvation rates. It seems therefore that another hitherto neglected factor may be of greater importance than believed in recent years: namely, the oxygen tension as such. It is of interest in this connection to note that Füsser and Krüger (1951) concluded from their study of normal and carbon monoxide-poisoned *Planorbis corneus* and *Lymnaea stagnalis* that the diffusion hypothesis alone does not suffice to explain the oxygen consumption-oxygen tension relationships. They assume a direct influence of the oxygen concentration but do not elaborate the possibility in greater detail. We shall return to this point after having discussed the post-anaerobic respiration of our objects.

All four species of snails used in the present study showed a respiratory rebound and in all species it was characterized by its long duration. In this respect the snails resembled the clam *Sphaerium corneum* (Jatzenko, 1928). In some other invertebrates, such as insects and worms, the repayment period lasts a shorter time, but the percentage increase of the post-anaerobic respiration may be appreciably higher (Harnisch, 1936, 1948; von Brand, 1947).

The post-anaerobic oxygen consumption of the snails appeared more dependent

[3] It is realized that this statement may need revision if further evidence becomes available. If starvation reduces the enzyme content of the cells materially, or if one accepts the free-radical mechanism for enzymatic reactions, starvation may change diffusion.

on the tension than the pre-anaerobic consumption when expressed in absolute terms or in per cent of the pre-anaerobic consumption in 21 per cent oxygen. In this respect the present findings parallel those of Harnisch (1936), but they do not necessarily support his conclusions. In his opinion the above phenomenon indicates that normal and excess respiration are mediated through different enzyme systems. However, our data show that in aquatic snails the post-anaerobic oxygen consumption is raised over the pre-anaerobic rate by approximately the same percentage, regardless of the tension employed. This apparently hitherto unrecognized relationship leads inevitably to the picture of greater dependency of the rebound respiration on the tension than that shown by the normal respiration, if expressed in absolute terms or in per cent of the pre-anaerobic value shown in 21 per cent oxygen. It is simply a consequence of the fact that increasing a graded series of figures by the same percentage makes the difference between elevated and base figure the greater, the greater the magnitude of the base figure is. Evidently, then, the oxygen tension/oxygen consumption relationship does not give any information as to whether different enzyme systems are responsible for the pre- and post-anaerobic oxygen consumption.

Our data also obviously contradict von Buddenbrock's (1939) view that the greater dependency of the overshoot respiration is attributable to a shift in the critical point towards a higher oxygen tension owing to the increased rate shown in this period. However, our observations do raise some new questions which at present can be answered only tentatively.

The obvious first question is why the post-anaerobic respiration should be raised by the same percentage over a wide range of tensions. Apparently, the degree of anaerobic stress plays only a minor role. The percentage increase was, within admittedly rather wide limits of variations, similar regardless of the temperature at which the specimens of one species had been exposed for an equal period to anaerobiosis. There is general agreement that the overshoot phenomenon is due to the accumulation of end-products of anaerobic metabolism. The increase in substrates would lead to an increased probability of enzyme and substrate molecules colliding (Zimmerman, 1949). The long period of repayment seems to indicate that a regulatory mechanism exists in snails which releases the "abnormal" substrates only slowly to the oxidative processes. This process might be geared to the rate of oxidations involving the normal substrates. Such an assumption would explain the phenomena observed.

Another very interesting question is the explanation of the physiological mechanism by which snails and other organisms are able to raise their post-anaerobic respiratory rate at those tensions where the normal respiration declines. This quite common phenomenon seems, surprisingly enough, never to have been discussed. It is a curious phenomenon because it indicates a regulatory mechanism that comes into play only post-anaerobically but not when the snail is exposed pre-anaerobically to oxygen concentrations allowing only 50 to 60 per cent of the normal maximal rate of respiration. If diffusion of oxygen into the body were the only limiting factor pre-anaerobically, one would obviously have to look for factors that would facilitate a greater influx of oxygen post-anaerobically. Such factors may be an increased surface by maximal extension of the foot, a facilitated oxygen release by the blood due to a Bohr effect, a steeper oxygen gradient in the

tissue layers adjacent to the diffusion surfaces owing to the accumulation of substrates, or due to an increased enzyme concentration. Such factors might well be important and possibly suffice to explain the phenomenon. Unfortunately, a highly organized animal, such as a snail, represents so complex a system that a complete analysis of these factors, experimentally or mathematically, would appear to be extremely difficult, if not impossible. It seems therefore appropriate to point out that at least one other alternative exists.

It is, for example, possible to discuss the present findings from a standpoint already touched upon above, namely, to raise the question whether the importance of the oxygen tension as such may not be greater than is usually suspected. Assuming that at least one enzymatic key-process of the cellular oxidation mechanism operates more efficiently at higher than at lower oxygen tensions, the following picture would appear. The relative inefficiency of a changed metabolic level in influencing the critical point of the pre-anaerobic oxygen consumption/oxygen tension relationship would be understandable without any auxiliary hypothesis since the influence of the tension would remain the same regardless of the temperature employed or the nutritional state of the snails. A necessary implication of this view would be that even at low tensions (low within limits, of course) the decrease of the pre-anaerobic oxygen consumption would not be due to the establishment of an anoxic zone in the deeper tissue layers. Even these tissues, like all the others, would get enough oxygen to allow a certain sub-optimal oxidative activity, the actual level of which would be determined on the one hand by the intracellular oxygen tension as influenced by the external tension, and on the other hand, by the availability of substrates. It may be pointed out in this connection that the fluoroacetate inhibition of citrate utilization in pigeon breast muscle is profoundly influenced by the oxygen tension (Massey and Rogers, 1951). Furthermore, Lehmann (1935) has found that succinic dehydrogenase was most active at oxygen tensions of 44 to 56 mm. Hg, while the activity significantly decreased at lower tensions. Finally, Schade and Levy (1949) have found in potato tissues, besides cytochrome oxidase, a terminal oxidase which was markedly sensitive to lowered oxygen tensions.

Insofar as post-anaerobic conditions are concerned, the increase in substrates and their gradual release to the oxidative processes would allow an increased oxygen consumption of all tissues and hence lead to an increase in the over-all oxygen consumption at all tensions where the above requirement is fulfilled, namely, that diffusion limitations would not be so severe as to lead to the establishment of an anoxic zone. The mechanism of the post-anaerobic increase in rate would hence be reduced to the accumulation of anaerobic metabolic end-products. The occurrence of the same percentage increase in post-anaerobic respiration over a wide range of tensions could also be understood better because it can be visualized that under otherwise identical conditions, the increased substrates and their gradual release may have, percentage wise, the same stimulatory effect on optimal and sub-optimal oxidative activity.

It should be understood that the above explanation is proposed only as a possible working hypothesis. It is realized that the available evidence does not constitute a definite proof. Furthermore, the above hypothesis is not mutually exclusive with the diffusion hypothesis. In any animal diffusion must become the

limiting factor if the tension is lowered sufficiently. The critical tension will vary from species to species, probably being highest in bulky animals lacking a circulatory system, like the actinians. Conversely, it is indisputable that somewhere along the line of oxygen tensions a critical point must be reached for any oxidation process below which it cannot function with full effectiveness. Insofar as the snail species studied are concerned, it is probable that both components enter the picture. Least affected by diffusion difficulties and hence most subject to the direct influence of tension seems to be *Australorbis glabratus,* while in *Helisoma duryi* and *Lymnaea stagnalis* diffusion difficulties seem to play a greater role as evidenced by the influence of the metabolic level on the critical point.

Summary

1. The respiration of four species of fresh water snails was somewhat dependent on the oxygen tension below 21 per cent oxygen with only minor differences among the various species.

2. The degree of dependency was influenced little, if any, by alteration of the metabolic rate of *Australorbis glabratus,* but some changes were obtained in the cases of *Helisoma duryi* and *Lymnaea stagnalis.*

3. All species showed a long lasting respiratory rebound after 16 hours anaerobiosis. The post-anaerobic respiration was more dependent on the oxygen tension than the pre-anaerobic respiration if referred to the normal rate shown at 21 per cent oxygen. But if the post-anaerobic rates were compared with the rates sustained pre-anaerobically at an identical oxygen tension, an approximately equal percentage increase was observed over a wide range of tensions.

4. The implications of these observations and auxiliary observations dealing with the rate of the heart-beat under various conditions are discussed insofar as they shed light on the mechanism of the pre- and post-anaerobic respiration. It is concluded that diffusion alone cannot be the sole limiting factor that reduces the over-all oxygen consumption when the tension is lowered below a critical point, and the idea is discussed that the oxygen tension as such may be more important for certain cellular processes than usually assumed.

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MITOTIC EFFECTS OF PROLONGED IRRADIATION WITH LOW-INTENSITY GAMMA RAYS ON THE CHORTOPHAGA NEUROBLAST

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When cells are subjected to given doses of ionizing radiation delivered at very low rates, the detectable effects on mitotic activity, as measured at the end of the treatment period, resemble those obtained from smaller doses administered at higher intensities (Carlson, Snyder and Hollaender, 1949; Carlson, 1950). This is due, apparently, to the capacity of living cells to recover from the more immediate physical and chemical changes induced by irradiation even while treatment is in progress. At the end of a short treatment period the reduced mitotic activity will represent virtually all the potential mitotic effect produced, because little recovery from these initial effects will have occurred. At the end of a long period of irradiation, on the other hand, the decreased mitotic activity will be the result of only the physico-chemical changes produced in the later part of the treatment period, those induced earlier having undergone recovery before the period of observation.

The main purpose of the present study was to investigate the mitotic effect of prolonged low-intensity treatment and to determine whether a balance between effect and recovery would be established and maintained at about the same level over much of the extended periods during which treatment was in progress. If such a balance were established, the detectable mitotic effects would be approximately the same at the ends of a wide range of treatment periods for a given dosage rate.

Materials and Methods

Eggs of *Chortophaga viridifasciata* (DeGeer) were used in all experiments. Because the times of treatment ranged from two to six days, it was necessary to start with embryos sufficiently young so that at the end of treatment they would be at the optimum age (14 days at 26° C.) for studying the mitotic activity of the neuroblasts.

The apparatus used in this study to irradiate the grasshopper eggs with γ rays is shown in Figure 1. The source (B) consisted of activated cobalt-aluminum alloy in the form of a hollow cylinder 8 inches long and 2 inches in diameter with walls 0.03 inch thick. This was enclosed in a cylindrical lead pig (A), the walls and lid of which each measured 2 inches in thickness. A cylinder of polystyrene

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[2] Contribution No. 71 from the Department of Zoology and Entomology of The University of Tennessee.

(C), bored from one end to a depth of 5½ inches with a ¾-inch bit, fitted snugly inside the source. Four polystyrene vessels (E), which were threaded so that they could be screwed to one another and to a polystyrene plug (D) held the eggs during treatment. The interior dimensions of each vessel, when screwed together, were approximately ½ inch in diameter and ¼ inch in depth. Treatment was begun by inserting the joined vessels and plug, vessels first, into the bore of the polystyrene cylinder (C). When in this position, the material to be treated was situated no more than one inch above or below the middle of the source, which extended at least three inches beyond the vessels at either end. Treatment was terminated by withdrawing the plug and attached vessels.

FIGURE 1. Gamma source and accessories. A, lead pig; B, cobalt-aluminum alloy source; C, polystyrene cylinder; D, polystyrene plug; E, polystyrene vessels; F, polystyrene vessel (enlarged) containing grasshopper eggs.

This type of source has several advantages (see Sheppard, 1949). The dosage rate can be determined to an accuracy of about 2%. The long half life of the cobalt (5.3 years) makes it possible to use a given source over a period of several months with very little decrease in the dosage rate. Because the source is cylindrical and its interior is filled with polystyrene, the radiation field is quite uniform throughout the space containing the biological material. Further, low-intensity treatment can be maintained at a constant level over long periods of time inside a protective lead container occupying less than a square foot of the floor.

Ideally, all the space in the specimen vessels not occupied by the biological material should contain water. Since the complete immersion of grasshopper eggs in water affects mitoses very adversely, presumably because of the reduced

exchange of oxygen and carbon dioxide between the egg and the surrounding medium, only enough moisture was placed in each vessel to keep the eggs from drying out. The pocket of air in each vessel is so small (about 0.05 cubic inch) that it should have no appreciable effect on the amount of radiation received by the eggs. The vessels were opened for a very brief period every 48 hours in the 3.4 r/hour experiment and twice daily in the 0.80 r/hour experiments during the treatment period to prevent accumulation of excessive amounts of carbon dioxide and depletion of oxygen in the vessel containing the eggs. In all experiments, irradiated and control embryos were from the same egg pods and were handled identically except for the presence or absence of the activated cobalt-aluminum cylinder in the pig.

The source was calibrated by substituting for the plug and vessels in the polystyrene bore a thimble chamber of the type designed by Darden and Sheppard (1951) and comparing the intensity of ionization with that measured when the same chamber was exposed to a radium source of known mass.

At the end of the irradiation period, which lasted for 2, 4 or 6 days, control and treated eggs were removed from the vessels and made into hanging-drop preparations by the method described previously (Carlson and Hollaender, 1944; Carlson, 1946).

Except in the final experiment, in which the numbers of middle and late prophases were also recorded, mitotic activities of the treated and control embryos were determined by counting the mid-mitotic [3] neuroblasts from the first maxillary through the first abdominal segments at 22-minute intervals. The average time required by the neuroblast to pass through mid-mitosis is about 22 minutes; therefore, the total of a number of such counts approximates the total number of cells undergoing mitosis within the period of time involved. Information on the number of counts made, the time after treatment of the initial count, the dosage rate, and the time of treatment, which differed from one experiment to another, is included with the description of the individual experiments.

Observations and Interpretation

Dosage rate of 3.4 r/hour

The mitotic effects of treatment for 6 days (143–145 hours) at a dosage rate of 3.4 r/hour are shown in Figure 2. The first count was made at an average of 16 minutes [4] after removal of the embryos from the source; subsequent counts were made at 22-minute intervals. Except for the second, or 38-minute, counting period, the number of mid-mitotic cells is reduced by this treatment to approximately 0.4 of normal. This is in striking contrast to what would have been obtained, if the same total dose—490 r—had been delivered in a few minutes instead of 6 days. Then the mitotic activity of the irradiated cells would have been

[3] This refers to cells in prometaphase, metaphase, or anaphase, *i.e.*, those between the breakdown of the nuclear membrane at the end of prophase and the loss of distinctness in the appearance of the chromosomes that marks the advent of telophase.

[4] The times after irradiation at which the initial counts were made differ from experiment to experiment, because we did not recognize the importance of an immediate first count until the early experiments had been completed.

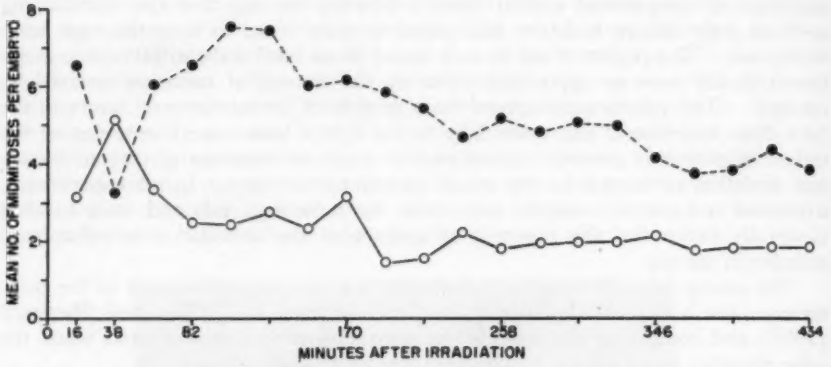

FIGURE 2. Dosage rate, 3.4 r/hour. Duration of treatment, 143–145 hours; dose, 489.6 r. ○, irradiated cells; ●, control cells

reduced to zero within an hour after treatment and would have remained at that level for about 5 hours. This demonstrates clearly the capacity of cells to undergo recovery from mitotic effects of radiation during the period of treatment.

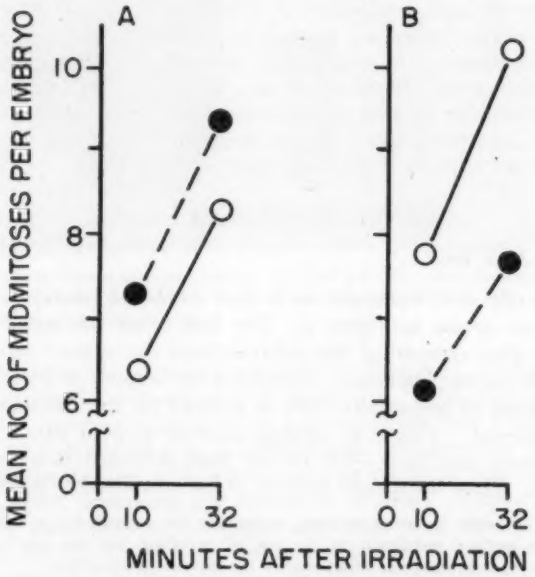

FIGURE 3. Dosage rate, 0.80 r/hour. A, duration of treatment, 44–51 hours; approximate dose, 35–41 r. B, duration of treatment, 92–99 hours; approximate dose, 74–79 r. ○, irradiated cells; ●, control cells

TABLE I

Summary of data and biometrical analysis

Dose rate (r/hr.)	Duration of irradiation (hrs.)	Approximate dose (r)	No. of embryos		No. of counting periods	Stages counted	Per cent level of significance		Least significant difference (5% level) between treated and control embryos for any one counting period*	Standard error of a single count*	Degrees of freedom for error
			Irradiated	Control			Between treated and control counts averaged over all counting periods	For interaction between treatment and counting period			
3.4	143–145	490	16	16	20	Mid-mitosis	<0.1	<0.1	0.46	0.67	562
0.80	44–51	35–41	31	31	2	Mid-mitosis	20	>50	0.30	0.60	58
	92–99	74–79	20	20	2	Mid-mitosis	3.6	43	0.42	0.64	38
	144	115	11	11	16	Mid-mitosis	>50	<0.1	0.54	0.65	300
	144	115	15	14	4	Middle prophase	42	>50	0.76	0.81	73
						Late prophase	<0.1	9.7	0.53	0.68	73
						Mid-mitosis	>50	18	0.60	0.89	77

* These figures should not be compared with the mean counts shown on the graphs, but with their approximate square roots. See text footnote 5.

The statistical analysis of the 3.4 r/hour data [8] summarized in Table I shows that the average numbers of mid-mitotic cells in treated and control embryos averaged for the whole counting period differ significantly at the 0.1% level of probability. Interaction is indicated by the fact that differences between treated and control embryos vary significantly at the 0.1% level from count to count.

Dosage rate of 0.80 r/hour

After two-day exposures at this dosage rate, mid-mitotic counts at 10 and 32 minutes after irradiation (Fig. 3A) were not significantly different from those of the control embryos, with respect to the counts averaged over all counting periods or the interaction (Table I). After four-day exposures, however, the mid-mitotic counts at both 10 and 32 minutes after irradiation were higher than in the controls (Fig. 3B). Biometrical analysis indicates a significant difference at the 3.6% level for the counts averaged over all counting periods and for the second counts alone at the 5% level, but not for the first count or for interaction (Table I).

Some preliminary 6-day exposures at this dosage rate also gave initial mid-mitotic counts that were distinctly higher than those of the control embryos. This unexpected result, it was reasoned, could have resulted from any of three factors. (1) Prolonged low-intensity radiation might have a stimulating effect on mitosis. By this we mean that a more rapid passage of cells through the stages of the mitotic cycle between the end of anaphase and the beginning of prometaphase, namely, telophase, interphase, or prophase, might throw a proportionally greater number of the cells into mid-mitosis. (2) The progress of cells through the stage being counted, namely, mid-mitosis, might have been retarded, so that the same cell was included in successive counts, thus giving a false picture of increased mitotic activity. (3) Cells might have been retarded in late prophase, accumulating there in abnormally large numbers, and then progressing simultaneously into mid-mitosis after removal from the field of radiation.

With these possibilities in mind an experiment was set up to determine whether or not this initial "stimulating" effect was the result of counting the same mid-mitotic cell twice in successive counts. Treatment was at the rate of 0.80 r/hour for 6 days (total dose, 115.2 r). Sixteen successive counts were made at 22-minute intervals beginning 22 minutes after the end of radiation. During each observation period, neuroblasts in mid-mitosis were not only counted but also the exact stage and the location of each cell were recorded. From these data it was possible to correct for all cases in which a cell was counted twice by eliminating the second record. The only counting period for which this correction averaged more than 0.3 count per embryo was the second. Corrections for that period reduced the average count from 13.3 to 11.2 per irradiated embryo and from 6.0 to 4.55 per control embryo. The corrected results are used in Figure 4.

Biometrical analysis (Table I) indicates that there is no significant difference in the mitotic activity of the treated and control embryos for the period of observation taken as a whole. Interaction, however, is significant at the 0.1% level. The means of the 22- and 44-minute counts (Fig. 4) of the treated cells are sig-

[8] All analyses were based on the square root of counts, because this transformation makes variances approximately uniform.

nificantly higher than those of the untreated cells while the means of the 110-, 132-, and 154-minute counts of the treated cells are significantly lower than those of the control cells at the 5% level. The possibility that the higher initial number of mid-mitotic cells in the treated than in the control embryos resulting from counting the same cell twice is, therefore, eliminated.

To determine whether the high initial number of mid-mitotic cells results from simultaneous recovery and mitotic progression of an abnormally large number of cells retarded at middle and late prophase by the irradiation, an experiment was

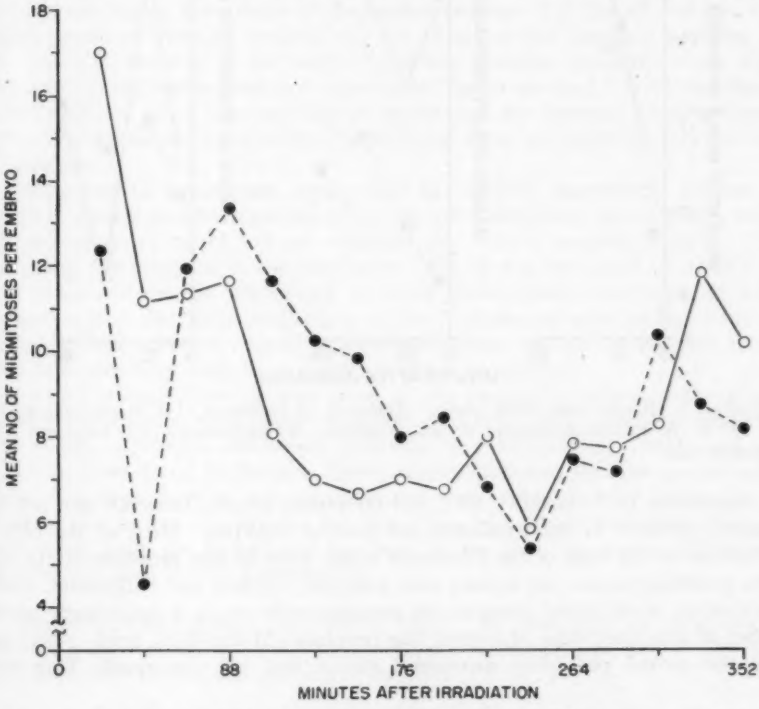

FIGURE 4. Dosage rate, 0.80 r/hour. Duration of treatment, 144 hours; dose, 115 r. ○, irradiated cells; ●, control cells

run in which the counts were made of middle prophases, late prophases and mid-mitoses. The first count was made as soon after irradiation as the material could be prepared for observation, *i.e.*, 4.5–6.0 minutes after removal from the radiation field. Three additional counts were made 22, 44, and 66 minutes after the end of radiation.

The results are shown graphically in Figure 5. The 22-minute mid-mitotic count, like that in the preceding experiment, is considerably higher than the corresponding control count. The difference is significant at the 8.2% level (Table II).

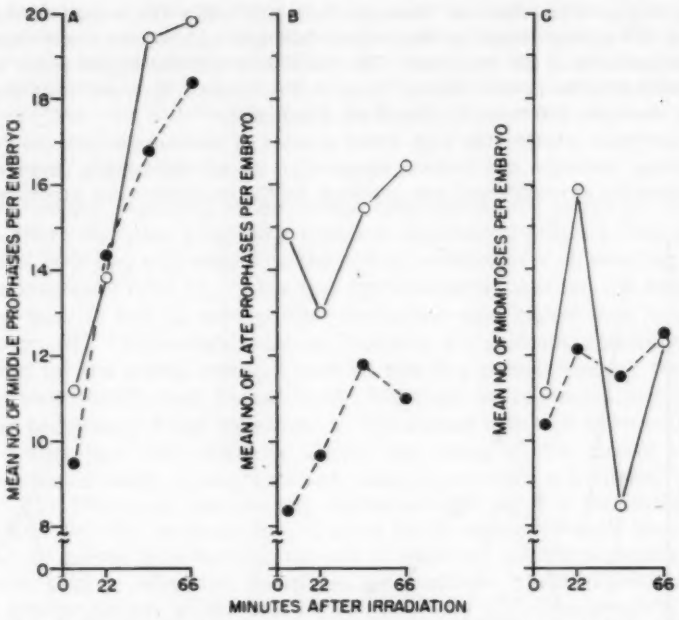

FIGURE 5. Dosage rate, 0.80 r/hour. Duration of treatment, 144 hours; approximate dose, 115 r. A, middle prophases; B, late prophases; C, mid-mitoses. ○, irradiated cells; ●, control cells

The immediate (4.5- to 6.0-), 44-, and 66-minute counts, however, are not significantly different in the irradiated and control embryos. Most of the cells in mid-mitosis at the time of the 22-minute count were in late prophase at the time of the preceding count (see mitotic time schedule, Carlson and Hollaender, 1948). Examination of the initial count of late prophase cells shows a significantly greater number of irradiated than of control late prophases at the 0.1% level. This supports the second possibility mentioned earlier that the abnormally high early

TABLE II

Comparison of treated and control counts of middle prophases, late prophases, and mid-mitoses for four counting periods following 144 hours of irradiation at 0.80 r/hour

Minutes after irradiation	Per cent level of significance between irradiated and control embryos		
	Middle prophase	Late prophase	Mid-mitosis
4.5–6.0	55	<0.1	65
22	88	5.5	8.2
44	46	8.5	21
66	64	0.5	87

mid-mitotic count results from the gradual accumulation of many cells in late prophase during the long irradiation period. That the inhibitory effect of the irradiation on late prophase cells persists in some measure even as late as 44 and 66 minutes after treatment is evidenced by the significantly greater numbers (at the 8.5 and 0.5% levels, respectively) of late prophases at these counting periods.

The irradiated middle prophases are not significantly different from the controls with respect to averages over all counting periods, interaction, or any of the four counts.

It seems worth while to point out that the mean counts (Fig. 5) in treated embryos are higher than those of the control embryos (1) for all but one of the middle prophase counting periods, (2) for all of the late prophase counting periods, and (3) for two of the four mid-mitotic counting periods. Even though only three of the higher ones are *significantly* higher at the 5.5 level, the fact that three-fourths of all of them are higher emphasizes the tendency of neuroblasts to accumulate in certain parts of the mitotic cycle when subjected to very low doses of radiation.

These results demonstrate again what has become increasingly evident over the years during which the mitotic effects of irradiation have been studied, namely, that radiation can retard but not stimulate the mitotic progress of cells. It appears that any increase in the number of cells in a given stage of mitosis soon after treatment can be interpreted to result from either retardation of mitotic progress within that stage resulting in an accumulation of cells, or entry into that stage in a brief period of time of an abnormally large number of cells that accumulated in a preceding stage as a result of mitotic retardation.

Further substantiation is also given to the transitory nature of the physicochemical radiation-induced effects that may lead ultimately to mitotic retardation. The results of earlier ultraviolet (Carlson and Hollaender, 1945) and γ-ray (Carlson, Snyder and Hollaender, 1949) studies indicated that the smaller mitotic effect of a large dose of low, as compared with high, intensity irradiation was probably due to less opportunity for interaction of primary radiation effects. In the present study the dosage rate has been so low that the chance of interaction has probably been virtually eliminated.

The authors are greatly indebted to Dr. C. W. Sheppard for designing and calibrating the γ-radiation source for special use in this project, and to Dr. A. W. Kimball for the biometrical analysis of the data.

Summary

1. Prolonged treatment of *Chortophaga* neuroblasts with low-intensity γ radiation reduces mitotic activity much less than a comparable dose given at high intensity.

2. Treatment for 6 days at 3.4 r/hour reduced the mid-mitotic count to about 40% of normal for a period of about 7 hours following treatment. (The same dose administered in a few minutes would have reduced the mid-mitotic count to zero for about 5 hours beginning within an hour of the end of treatment.)

3. Continuous irradiation at 0.80 r/hour (1) failed to produce a significant mid-mitotic effect at the end of two days, (2) produced a significant *increase* in

the number of mid-mitoses through the second counting period at the end of four days, and (3) produced a significant *increase* in the number of mid-mitoses through the second counting period after 6 days of treatment.

4. The radiation-induced increase in the number of mid-mitoses immediately following four and six days of treatment is shown to result, not from a stimulating effect of the radiation on mitotic activity, but from the simultaneous progression into mid-mitosis of neuroblasts that have accumulated in late prophase as a result of the inhibiting effect of the radiation on this stage.

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THE EFFECT OF VARIOUS SUSPENSION MEDIA ON THE ACTIVITY OF CHOLINESTERASE FROM FLIES

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The cholinesterases (ChE's) of insects merit intensive study both because of their importance for normal nervous function in these animals and on account of their consequent role in the mode of action of certain highly effective insecticides (Roeder *et al.*, 1947; Dubois and Mangun, 1947; Chadwick and Hill, 1947; Roeder, 1948; etc.). Moreover, it is to be expected that the comparative study of enzymes of this type from diverse species will speed progress toward the definition and understanding of the significant properties of ChE's generally. We have therefore investigated the acetylcholine (ACh)-splitting mechanism in the nervous tissue of houseflies.

Several properties of this system have already been reported by Metcalf and March (1949, 1950) and by Babers and Pratt (1950, 1951). In their experiments with fly-head brei, these workers adopted suspension media commonly used with vertebrate ChE's and found such solutions compatible with the insect enzyme. Our assays were begun similarly, but it soon became apparent that maximal activity of fly-head ChE is not obtained under these conditions. It has seemed worthwhile for this reason to make a systematic study of the various factors which influence the rate at which ACh is hydrolyzed by fly-head brei. The present report is concerned mainly with the effects of variation in salt concentration. Some observations with solutions of glycerol and sucrose are included.

Material and Methods

1. *Material.* The data below refer to a strain of houseflies (*Musca domestica* L.) that was obtained early in 1948 from the testing stock of a commercial laboratory and that has been maintained in mass culture without exposure to insecticides or other toxicants. The larvae were grown at 32 degrees C. and approximately 30 per cent relative humidity (room conditions), in ground horsemeat placed between layers of sterile sawdust. The resulting pupae were blown free of sawdust and held in jars for emergence. Before mating had taken place, the adults were separated according to sex, and were kept in cages until use, with dry sugar, dry whole milk, and a source of water. Breeding stocks were housed in other cages and, in addition to the usual food and water, were supplied daily with ground horsemeat as an oviposition medium. The eggs or newly hatched larvae were then transferred to rearing jars. Under these conditions, development from egg to adult required 7 or 8 days.

2. *Preparation of tissues.* Adult flies of known age and sex were anesthetized with carbon dioxide gas. The heads were removed with fine scissors, counted into tared weighing bottles, weighed, ground, diluted to the desired concentration,

and assayed. Alternatively, batches of heads or whole flies were quick-frozen and stored indefinitely at −15 degrees C.; after thawing, they were processed at once. When necessary, tissue suspensions were held overnight or for longer periods in a refrigerator at 2 to 3 degrees C.

Breis were prepared from the heads by grinding them in a Pyrex test tube with 2 to 5 ml. of the desired suspension medium. A stainless steel pestle, rotated by a drill press at 700 r.p.m., provided a convenient and durable modification of the original all-glass Potter-Elvehjem homogenizer. Small samples, containing from 1 to 20 heads, were ground at room temperature for 30 to 35 seconds. Larger samples, up to 500 heads, were ground for longer periods, in a test tube surrounded by cracked ice. No abrasive was added.

3. *Method of assay.* Production of acid from ACh.Br was measured as proposed by Glick (1937), by means of titration with standard alkali at "constant" pH, using the Beckman Model G meter as a null instrument. The following conditions were adopted: tissue concentration, one head per ml.; final volume of sample, 20 ml.; reaction vessel, 50 ml. beaker; temperature, 25.0 degrees C.; pH, 8.0; rate of addition of 0.1 N NaOH during assay, 0.01 or 0.02 ml. as often as required to keep pH from dropping below 8.0; duration of measurement, approximately 15 minutes; beaker contents agitated by hand every 15 to 30 seconds; initial concentration of ACh.Br in assay mixture, 0.015 *M*.

Concentrated breis, containing 10 ground heads per ml., were made up in water, and two-ml. aliquots were diluted with stock reagent and water so as to provide a tissue concentration of one head per ml. in the desired strength of reagent. The reagent and sample containers were kept partially immersed on a shelf in the constant temperature bath. After addition of ACh.Br to the sample, pH was rapidly adjusted to slightly above 8.0 with 0.1 N NaOH or HCl. The pH was then allowed to fall to 8.0, at which time a stopwatch was started and 0.01 or 0.02 ml. of 0.1 N NaOH added by pipette. This raised pH to a value between 8.0 and (in extreme cases) 8.5; it was again allowed to fall, with stirring, and when 8.0 had been reached again the time was recorded to the nearest second and another 0.01 or 0.02 ml. of 0.1 N NaOH added. This routine was repeated as often as necessary for approximately 15 minutes. The rate of production of acid was then calculated from the elapsed time and the total volume of alkali used.

The reproducibility of the technique was measured by determining the activity of 20 aliquots, equivalent to 20 heads each, from a single sample of brei, which was suspended in a buffered solution of the following composition: NaCl, 26.30 gm.; KH_2PO_4, 3.85 gm.; NaOH, 1.00 gm.; H_2O, to one liter; approximate normality with respect to cations, 0.5; pH adjusted to 8.0. The average ChE activity of this preparation, after correction for acid produced from other sources, was 5.15 ± 0.23 micromoles ACh.Br hydrolyzed per head per hour. Thus, the coefficient of variability amounted to ± 4.5 per cent. Repetitions in unbuffered 0.5 N $MgCl_2$ and 0.5 N NaCl with 16 aliquots each gave a coefficient of ± 2.8 per cent in both cases. Variation was of the same order when 20-head aliquots were individually prepared. Such a test in buffer with 20 samples from a batch of male flies 5 to 6 days old yielded a coefficient of variability of ± 3.8 per cent.

4. *Reagents.* With the exception of glycerin, which was of USP quality, 98 per cent pure, reagents were of CP grade. Stock solutions were prepared with

de-ionized water. The concentrations of the chlorides were checked by titration with 0.1 N $AgNO_3$. Alkali employed for titrating acid that was produced by the samples was rechecked against standard 0.1 N HCl at intervals. The loss of alkalinity amounted to less than 2 per cent in several months. ACh.Br, recrystallized from the Paragon or Matheson product, was made up fresh daily at 0.15 *M* concentration in de-ionized water.

5. *Treatment of data.* The rates reported represent enzymic hydrolysis, the

TABLE I

ChE activity of fly-head suspensions as a function of concentration of single salts, glycerol or sucrose

NaCl												
Normality	0	0.0001	0.001	0.01	0.06	0.12	0.24	0.50	1.08	1.62	2.16	3.24
Activity in per cent*												
Average	100	105	107	122	188	196	222	242	238	213	190	121
Maximum	—	106	110	127	216	209	237	267	255	235	201	128
Minimum	—	104	105	118	177	174	200	200	221	192	182	114
Number of samples	7	5	5	5	5	5	5	5	5	5	5	5
Correction**	−0.56	−0.56	−0.56	−0.55	−0.52	−0.46	−0.50	−0.33	−0.26	−0.25	−0.24	−0.22
KCl												
Normality	0	0.036	0.072	0.15	0.27	0.54	0.81	1.08	1.62			
Activity in per cent*												
Average	100	168	192	218	242	248	247	231	204			
Maximum	—	183	212	241	263	264	262	249	218			
Minimum	—	150	171	162	209	221	230	217	170			
Number of samples	7	5	5	5	5	6	5	5	7			
Correction**	−0.56	−0.41	−0.41	−0.37	−0.33	−0.31	−0.27	−0.26	−0.24			
$MgCl_2$												
Normality	0	0.0001	0.001	0.01	0.058	0.115	0.23	0.46	0.92	1.84	3.68	
Activity in per cent*												
Average	100	102	118	162	241	255	257	271	268	217	38	
Maximum	—	106	121	164	255	268	279	296	277	235	47	
Minimum	—	96	114	157	225	230	225	252	257	194	30	
Number of samples	5	5	5	5	5	5	5	5	5	5	5	
Correction**	−0.56	−0.56	−0.55	−0.47	−0.29	−0.29	−0.32	−0.34	−0.46	−0.70	−1.19	
$CaCl_2$												
Normality	0	0.051	0.102	0.20	0.41	0.81	1.63	3.26				
Activity in per cent*												
Average	100	187	197	229	242	199	101	nil				
Maximum	—	228	241	250	267	219	128	−1				
Minimum	—	198	207	190	212	190	112	−16				
Number of samples	5	5	5	5	5	5	5	5				
Correction**	−0.56	−0.39	−0.41	−0.42	−0.46	−0.54	−0.71	−1.22				
$NaNO_3$												
Normality	0	0.06	0.12	0.24	0.50	1.08	1.62	2.16	3.24			
Activity in per cent*												
Average	100	173	194	200	207	190	170	148	113			
Maximum	—	176	198	204	216	194	174	151	123			
Minimum	—	163	181	194	200	179	164	143	104			
Number of samples	4	4	5	5	5	5	5	5	5			
Correction**	−0.56	−0.38	−0.36	−0.35	−0.32	−0.32	−0.29	−0.25	−0.24			
Glycerol												
Molarity		0	0.125	0.25	0.50	1.00	2.00	4.00				
Average activity in per cent*		100	99	97	93	88	70	46				
Number of samples		2	2	2	2	2	2	2				
Correction**		−0.56	—	—	—	−0.51	−0.54	−0.50				
					(used	−0.56	for all	concentrations)				
Sucrose												
Molarity		0	0.03125	0.0625	0.125	0.25	0.45	0.90	1.35			
Average activity in per cent*		100	104	102	97	91	89	73	59			
Number of samples		2	2	2	2	2	2	2	2			
Correction**		−0.56	—	—	—	—	−0.47	—	−0.64			
					(used	−0.56	for all	concentrations)				

* Activity expressed in terms of that of the same breis in water = 100 per cent, as follows: for NaCl, 2.70 micromoles ACh·Br hydrolyzed per head per hr.; for KCl, 2.67; for $MgCl_2$, 2.23; for $CaCl_2$, 2.38; for $NaNO_3$, 2.45; for glycerol and sucrose, 2.44. Tissue concentration, one head per ml.

** Corrections (for acid produced from sources other than enzymic hydrolysis of ACh·Br) are in microequivalents per ml. per hr., and were applied to the raw data before calculation of per cent activity. All runs at 25.0 degrees C.; pH, 8.0.

total acid production having been corrected by subtraction of the fraction contributed by processes other than enzymic breakdown of substrate in the particular medium concerned. These correction values were determined by titration of 20-ml. aliquots that contained the usual concentrations of tissue and ACh.Br, but whose ChE activity had been destroyed by incubation of the stock brei overnight or longer with 1×10^{-5} *M* di-isopropyl fluorophosphate (DFP).

Net measurements of enzymic activity were converted into micromoles of ACh.Br hydrolyzed per mg. fresh weight *and* per head per hour. Our results are reported only on the latter basis. The head and body weight of flies varies with age, among other factors, and there are changes in proportion as well as amount of water and other constituents. Moreover, a large and likewise varying fraction of the total weight consists of cuticle, which is presumably free of ChE activity. In contrast with these variations, ChE activity per head remains relatively constant, at a somewhat different level in each sex, after the first day or two of adult life. On this account, it has been advantageous to make comparisons on a per-head basis rather than with reference to fresh or dry weight, or protein or nitrogen content. The average fresh weight of a head in most of our samples has been between 1.2 and 2.9 mg. Dry weight in different batches ranged from 0.33 to 0.65 mg., and from 22 to 36 per cent of the total.

Results

Table I shows ChE activities of fly-head suspensions as affected by various concentrations of single salts, glycerol or sucrose. Comparison has been simplified by expressing the average rates as percentages of the activity found for the same breis in the absence of added reagent. Such treatment of the data is considered proper because the degree of activation or depression observed with a given concentration of reagent, although somewhat variable from one suspension to another, was found to be independent of the absolute level of activity of the preparation. The outcome of a fresh comparison of the salts at nearly optimal concentrations, in which all runs were made with aliquots of the same stock brei, is shown in Table II. Not listed is a single test with 0.5 N $(NH_4)_2SO_4$, in which ChE activity was about twice that of the same suspension in water. This salt solution was too strongly buffered to permit satisfactory measurement of the necessary correction. Results of a few other experiments are cited in the discussion.

Table II

Comparison of ChE activity of fly-head suspensions in 0.5 N solutions of single salts

Salt	NaCl	KCl	$MgCl_2$	$CaCl_2$
Activity in per cent*				
Average	212	201	229	208
Range	204–217	194–212	213–254	197–228
Number of observations	5	5	5	5

* Activity expressed in terms of that in water (1.78 micromoles ACh·Br hydrolyzed per head per hour) = 100 per cent. All determinations made on aliquots of the same brei, at 25.0 degrees C., pH 8.0.

Discussion

1. *Non-enzymic hydrolysis.* Under our conditions of measurement, at pH 8.0 and 25.0 degrees C., 0.015 *M* ACh.Br in buffered solution or in the higher concentrations of monovalent salts produced 0.22 to 0.24 microequivalents of acid per ml. per hr., in either the presence or absence of the usual concentration (one head per ml.) of DFP-inhibited tissue. These results compare reasonably well with values approximated by interpolation from the data of Augustinsson (1948) for non-enzymic hydrolysis of ACh.Cl and ACh.Br in bicarbonate buffer.

As may be seen from Table I, we observed and have used for correction considerably higher values in many of our experiments.

The observations with the salt solutions fall into two distinct groups. With the monovalent compounds, that portion of the total acid production that was not inhibited by DFP decreased more or less exponentially as salt concentration was increased. With the chlorides of divalent metals, on the contrary, an initial drop in acid production at low salt concentrations was followed by an increase that was related linearly to the concentration of salt.

In both cases, apparently, the higher rates at very low concentrations were the product of a technical shortcoming; namely, that addition of alkali during titration raised the pH of the medium temporarily and thereby accelerated the hydrolysis of ACh.Br. This effect was naturally more marked the smaller the buffering capacity of the medium; it was therefore greater in the more dilute solutions, where addition of 0.01 ml. of 0.1 N NaOH to a 20-ml. sample raised pH from 8.0 to 8.5 or occasionally even higher. Lower correction values were obtained with these dilute solutions when alkali was added in 0.0025 ml. amounts, but these results, of course, were not applicable to our experimental conditions.

The increase in acid production observed when concentration of $MgCl_2$ or $CaCl_2$ was above 0.1 N probably reflects a true increase in the rate of hydrolysis of ACh.Br. At pH 8.0 such solutions are quite strongly buffered, so that changes in pH on addition of alkali during titration become very small and cannot be held responsible for the effects noted. In the absence of substrate, tissue in 4.0 N $MgCl_2$ produced about 0.1 microequivalent of acid per ml. per hr. Ten times as much acid was released when substrate was present and tissue omitted. The increased hydrolysis in strong solutions of $MgCl_2$ is evidently not dependent on the presence of tissue, and it may be mentioned in passing that DFP-inhibited enzyme is not reactivated at all by incubation with either monovalent or divalent salts at 4.0 N concentration, even after several days. Other tests with 0.015 *M* ACh.Br in 4.0 N $MgCl_2$, where the loss of ester over periods of one to three hours in the presence of DFP-inhibited enzyme was determined chemically by the method of Hestrin (1949), indicated a hydrolysis rate of about 1.1 micromoles ACh.Br per ml. per hr. at 25.0 degrees C. and pH 8.0. These results may be compared with the correction value of 1.19 shown in Table I for 3.68 N $MgCl_2$.

The saddle-backed curve of correction values observed with the divalent salts seems, then, to owe its shape to a combination of two factors: (1) an inadequacy of procedure that resulted in slightly higher average pH during titration in very weak solutions; and (2) an opposing tendency toward acceleration of hydrolysis of ACh.Br as the concentration of divalent ions was increased. The latter process seems to be related linearly to salt concentration. We have assumed that similar

factors are concerned in the results with $CaCl_2$, although our attempt to analyze the situation has been confined to solutions containing $MgCl_2$.

The correction values obtained with glycerol and sucrose at all concentrations tested differed little from those observed in plain water, except for some indications of a trend toward higher rates of acid production at the higher concentrations of these compounds. This trend was more conspicuous in a few tests where substrate concentration was 0.06 *M* instead of the usual 0.015 *M*.

In concluding this section of the discussion, we would like to emphasize that, in some contrast with non-enzymic hydrolysis, the rate of breakdown of ACh.Br

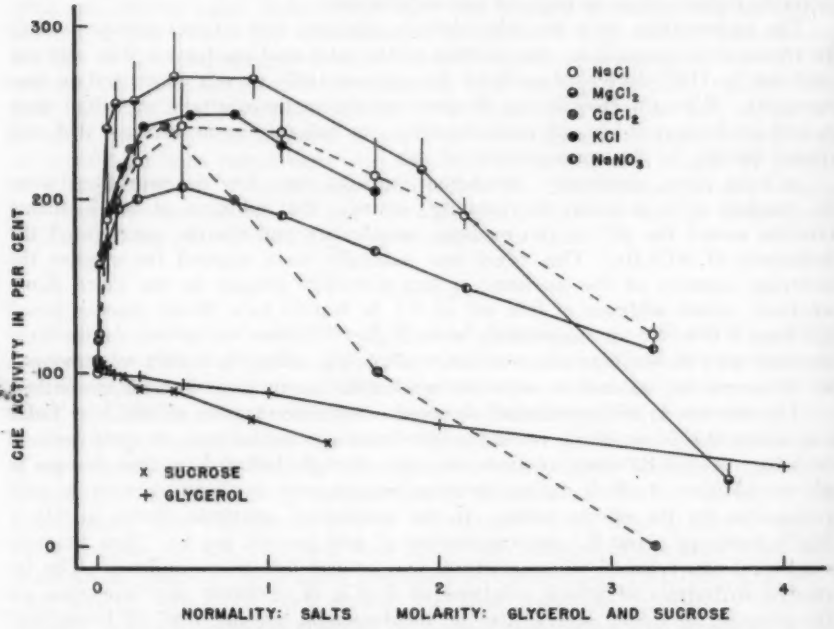

FIGURE 1. Relationship between ChE activity of fly heads and concentration of salt, glycerol or sucrose. Vertical bars indicate limits of variation encountered with NaCl and $MgCl_2$ solutions. For the range of variation with other salts, see Table I.

by ChE is but slightly affected by pH changes of as much as 0.5 unit in the neighborhood of pH 8.0, since activity at pH 8.5 is only 104 per cent of activity at 8.0. Use of correction values that were determined under conditions as nearly as possible identical with those prevailing during measurement of total activity therefore yields net results which should approximate closely the true rates of reaction between enzyme and substrate under the stated conditions. It is well to recognize that in certain circumstances the correction values may include acid production from a variety of sources other than what is usually thought of as non-enzymic hydrolysis, and that the rate of non-enzymic hydrolysis may be altered

several-fold as a result of details of technique or variation in the composition of the medium, even though pH and temperature are held within narrow limits.

2. *Variations in ChE activity.* From Tables I and II it is evident that added salt up to 0.5 to 1.0 N had a non-specific activating effect on fly-head ChE, and that this effect was reversed at still higher concentrations. With all the salts tested, the activity-concentration curves were quite similar when concentration was expressed in terms of normality, but the correspondence was less good when comparison was based on molarity or ionic strength. The maximal rate of enzymic hydrolysis was observed in all instances at salt concentrations ca. 0.5 N, and was some two to three times the value measured with water suspensions (Fig. 1).

Relationships of this type have not been reported for other ChE's, although certain scattered data are suggestive (*e.g.,* Alles and Hawes, 1940, with human erythrocytes). The somewhat discordant literature on the subject has been summarized by Augustinsson (1948), who ascribed the disagreements to the use of enzymes from varied sources. Undoubtedly, differences do exist in the response of various ChE's to the several ions, and for this reason it is perhaps surprising that Jandorf (1950), in studies of the esterase activity of chymotrypsin, obtained results strikingly similar to ours. However, in Jandorf's experiments activity seemed to be correlated with the ionic strength of the solutions.[1]

With the fly-head preparations, none of the ions tested appears to have the role of a necessary coenzyme. Aqueous suspensions, even as dilute as 0.1 head per ml., regularly yielded $Q_{ChE's}$ (at 25.0 degrees C.) of 15 to 25 mg. ACh split per 100 mg. fresh weight of tissue per hour. During dialysis against distilled or deionized water, loss of activity was slow, and not restored by added salts. Addition of any of the salts to these dialyzed preparations caused only the proportional change in activity already familiar from the studies with freshly prepared heads. Lack of specificity was likewise apparent in experiments with salt mixtures; for example:

Composition of medium:	water	0.5 N NaCl	0.5 N $MgCl_2$	0.25 N NaCl and 0.25 N $MgCl_2$
Activity in per cent:	100	258	275	263

In view of these various observations and the data in Tables I and II, it is unlikely that any ions present in the heads in a diffusible state (other than H^+ and OH^-) have an essential role in the activity of fly-head ChE. Nevertheless, slight differences in degree of activation were observed with some of the salts at equivalent concentrations. Thus, in both Tables I and II, as in the comparison cited in the preceding paragraph, it is apparent that the highest activity was found when $MgCl_2$ was present. The statistical significance of this difference was determined in an additional experiment. A stock brei was prepared, and 5 aliquots were run in water, 16 in 0.5 N NaCl, and another 16 in 0.5 N $MgCl_2$. The average (net) activity in water, taken as 100 per cent, was 2.32 micromoles ACh.Br hydrolyzed per head per hr. (range, 2.29 to 2.35). In 0.5 N $MgCl_2$, the

[1] Results similar in some respects to ours have been reported recently by C. van der Meer. 1953. Effect of calcium chloride on choline esterase. *Nature,* **171**: 78–79.

average ± the standard error was 256 ± 1.8 per cent; and in 0.5 N NaCl, 232 ± 1.6 per cent. The difference is 24 ± 2.5 per cent, or more than 9 times its standard error. Thus, there is little room for doubt that $MgCl_2$ does have a slightly greater potency for activation than does NaCl, and it is likely that further investigation would disclose equally significant though small differences among some of the other salts. It is also clear from our data that, after the peak at about 0.5 N is reached, the ChE activity of suspensions containing $CaCl_2$ declines much more rapidly than it does in other salt solutions. Some specificity in the effects of the different cations has therefore been demonstrated, but this question will need re-investigation when purified enzyme is available.

The mechanism of the non-specific activation seen with all the six salts tested is not known. Enzyme solubility can hardly be concerned, since filtrates or supernatants from aqueous suspensions of ground heads were found to contain well over 90 per cent of the total activity and responded to addition of salts exactly as did the crude brei. Besides, salt concentrations above 1.0 N depressed activity without necessarily precipitating or destroying equivalent amounts of enzyme. Thus, for instance, when a suspension was made 5.5 N with NaCl and centrifuged, 86 per cent of the original activity was recovered in the supernatant. (Comparison was made in 0.5 N NaCl.)

The depression of activity seen with the higher concentrations of salts is, then, very largely reversible, as is the activation noted at lower concentrations. Possibly the reduction in reaction rate at high concentrations could be explained on the basis that the dense cloud of cations around the enzyme tends to block electronegative sites that are concerned in formation of an enzyme-substrate complex, in accordance with the theory advanced by Wilson and Bergmann (1950a, 1950b). But it is not easy to relate the progressive activation by salts in concentrations up to 0.5 N to this concept, although an indication that the observed changes in activity may involve some alteration of the enzyme itself is provided by the observation that a 4-fold increase in substrate concentration failed to accelerate the rate of reaction in 3.12 N NaCl. This fact argues against the interpretation that the reduced activity observed at this salt concentration was the result of direct interference with access of substrate.

The question may be raised, in view of the analysis by Wilson and Bergmann (1950a), whether changes in the ionic composition of the suspension medium may not cause shifts in the pS optimum and thus complicate activity comparisons of the sort we have made. Experiments undertaken to settle this point by determining the substrate optimum in three media, *viz.*, de-ionized water, 0.5 N NaCl and 3.0 N NaCl, gave somewhat inconsistent results on replication. In each instance, 5 runs were made at each substrate concentration in the series 0.1, 0.03, 0.01, 0.003 and 0.001 *M*. Despite the variation encountered, a trend toward increase in substrate optimum with increasing concentration of salt may be discerned in the averages (Table III) and we would estimate the respective optima provisionally as follows: water, 0.0016 *M*; 0.5 N NaCl, 0.0025 *M*; 3.0 N NaCl, 0.005 *M*. The corresponding enzymic activities, interpolated from the average data obtained at the concentrations actually tested, were in the ratio of 100:242:129. These are so close to the ratios observed with the same salt concentrations in Table I, where all runs were made with ACh.Br 0.015 *M*, as to leave no doubt that any

shift in $pS_{opt.}$ with variation in salt concentration has played a negligible role in the comparisons with which this study is primarily concerned.

Glycerol was tested in order to provide a basis for future comparison of our observations with those of Babers and Pratt (1950), who used it in 30 per cent strength for preparation of stock breis. Since, in contrast to our experience with salts, we found that ChE activity was depressed progressively as concentration of glycerol was increased, we added a series of tests with sucrose, as another example of a non-electrolyte. Here, too, activity appeared to bear an inverse relationship to concentration, with the distinction that the slope of about -31 per cent per mole is appreciably steeper than the slope found with glycerol (about -14 per cent per mole).

TABLE III

Variation in activity of fly-head ChE in various media as a function of substrate concentration

Molar concentration of ACh·Br	0.001	0.003	0.01	0.03	0.10
	Average enzymic activity in micromoles per head per hour				
Suspension medium					
Water	2.46	2.32	1.89	1.99	2.12
0.5 N NaCl	4.82	4.91	4.40	3.51	2.49
3.0 N NaCl	2.96	3.22	2.99	2.86	2.37

Each series of observations is the mean of 5 replications.
All runs at 25.0 degrees C., pH 8.0.

Inasmuch as there seemed to be little likelihood that either of these compounds was acting as a chemical inhibitor, we were inclined to seek some physical mechanism, such as interference with diffusion of substrate toward or reaction products away from the active sites on the enzyme, as an explanation of the changes in activity observed. In such a case, the rate of hydrolysis might be expected, as a first approximation, to be related inversely to the viscosity of the medium. Unfortunately for this hypothesis, the viscosity of aqueous solutions of glycerol or sucrose does not vary linearly with concentration, whereas the depression of ChE activity did. Moreover, activity was depressed to about the same degree in 1.35 *M* sucrose, with a viscosity of about 5 centipoises, as in 4.0 *M* glycerol, whose viscosity is only some 2.5 centipoises. And in addition, as with the concentrated salt solutions, raising substrate concentration to 0.06 *M* did not yield greater activity in the presence of 4.0 *M* glycerol or 1.35 *M* sucrose; in fact, the rate of hydrolysis was reduced some 10 to 15 per cent in comparison with that obtained with 0.015 *M* ACh.Br, a result similar to what was seen with suspensions in plain water. Kodera (1928) also concluded that viscosity changes were not responsible for the inhibitory effect of gum arabic and soluble starch on the ChE's of human serum and red cells; and suggested that the test materials might have been adsorbed to the enzyme surface and thus have masked the active sites. His data, like ours with glycerol and sucrose, resemble the results with salt solutions to the extent that they indicate that the observed changes in activity are due to some alteration of

the enzyme. This could consist in blocking of the active sites as a consequence of adsorption or of some more fundamental alteration in the properties of the enzyme molecule.

Although further elucidation of the nature of these changes would be most desirable, it does not appear possible on the basis of present information. One may nevertheless draw the practical conclusion that, for experiments where maximal activity of fly-head ChE is desired, it will be well to have some salt present at about 0.5 N concentration, and to avoid the presence of compounds such as glycerol and sucrose.

The authors wish to acknowledge the kindness of Dr. Wm. E. Dove in supplying the pupae from which our fly culture was started.

Summary

1. The cholinesterase (ChE) activity of fly-head suspensions was measured titrimetrically at 25.0 degrees C. and pH 8.0 with acetylcholine bromide 0.015 *M* as substrate, as a function of the concentration of various single salts, glycerol, or sucrose. The species tested was *Musca domestica* L.

2. The salts, NaCl, KCl, $MgCl_2$, $CaCl_2$, $NaNO_3$, $(NH_4)_2SO_4$, at concentrations up to 0.5 to 1.0 N, had a non-specific activating effect. At still higher concentrations, activity was depressed progressively below the maximum, which was two to three times the value observed with water suspensions.

3. None of the ions tested was found essential to the activity of fly-head ChE. $MgCl_2$ was slightly but significantly more potent as an activator than NaCl.

4. Both glycerol and sucrose were depressant at all concentrations tested. The relationship was linear with concentration. For glycerol, the slope was about -14 per cent per mole and for sucrose, about -31 per cent per mole.

5. The depression of activity in the presence of higher concentrations of salts, glycerol or sucrose was not relieved by a four-fold increase in substrate concentration.

6. It is inferred that the changes in activity observed reflect alterations of an unknown nature in the properties of the enzyme, rather than direct interference with access of substrate to the active sites.

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ON FOOD AND FEEDING OF LARVAE OF THE AMERICAN OYSTER, C. VIRGINICA

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Feeding experiments have shown that larvae of the American oyster, *Crassostrea virginica*, are limited as to the types of food they can utilize, by factors other than the size of the micro-organism or particle. We have previously reported four species of marine bacteria and three species of flagellates that had no effect on the rate of growth of oyster larvae (Davis, 1950a). In one experiment, for example, in cultures that received sulfur bacteria as food, the larvae averaged only 94.05 μ in length at 14 days, a size not significantly different from that of larvae in unfed control cultures. Moreover, it was an increase of only about 20 μ over the initial size of early straight-hinged larvae which usually measure about 74–76 μ in length. In a pair of parallel cultures fed mixed phytoplankton composed chiefly of Chlorella, the larvae averaged 140.75 μ in length by the 14th day or had gained about 65 μ over their initial size.

The same series of experiments also showed that marine detritus, which we had collected from several sources, was not utilized by the larvae, and that organic media added to the larval cultures caused no increase in the rate of growth of the larvae.

On the basis of the above experiments it was reported (Davis, 1950a) that our mass culture of green phytoplankton consisting chiefly of Chlorella, which we shall refer to as "mixed Chlorella," was the best food for oyster larvae then available. It did not consistently induce a good rate of growth when fed to larvae during their earlier stages, but once the larvae had attained a size of approximately 125 μ they appeared to utilize the "mixed Chlorella" quite readily.

In the feeding experiments on oyster larvae reported in the following pages, we have tested nine additional species of marine bacteria, isolated from the mud of Milford Harbor by Dr. Burkholder of Yale University; six more species of marine flagellates, including some of the same varieties used by Bruce, Knight and Parke (1940), obtained from Dr. Russell of the Plymouth Laboratory, England; and a bacteria-free culture of *Chlorella* sp. isolated from our mixed phytoplankton culture by Dr. Ralph Lewin of Yale University. These feeding experiments were conducted during the winter and early spring using oysters brought to spawning condition by the method described by Loosanoff (1945) and by Loosanoff and Davis (1952).

To insure uniformity in size, quality, and number of larvae in the different culture jars of a series, at the beginning of an experiment, and except for foods, to provide as nearly equal treatment of all cultures as possible, the following procedure has been made standard practice.

To obtain fertilized eggs, the conditioned oysters were placed in spawning dishes, filled with sea water that had been filtered through cotton to remove debris

and most planktonic forms, and spawning was induced by adding small quantities of sperm suspension and raising the temperature to about 30.0° C. The eggs of *C. virginica,* because of their small size, cannot readily be separated from excess sperm, as can those of *Venus mercenaria* (Loosanoff and Davis, 1950). The spawning females were, therefore, separated from the males and placed in spawning dishes containing only a slight excess of sperm.

The fertilized eggs were collected in a tall narrow jar by screening the contents of the spawning dishes through stainless steel screens having 100 meshes per inch. This screen allowed the fertilized eggs to pass through unharmed but retained larger debris and feces expelled by the oysters during spawning. Since we wished to have 5000 larvae per liter in our experimental cultures, the egg suspension in the tall narrow jar was thoroughly stirred with a perforated plastic plunger to insure uniform distribution of the eggs and a sample was withdrawn and the number of fertilized eggs per ml. determined.

Only rarely did 100 per cent of the fertilized eggs develop into normal straight-hinged veliger larvae, but if less than 50 per cent developed normally, we discarded the larvae. It became our practice, therefore, to introduce enough fertilized eggs into each culture jar to give approximately twice the desired number of larvae if all the eggs should develop normally, *i.e.,* about 10,000 fertilized eggs per liter. The 20-liter earthenware culture jars were then filled with cotton-filtered sea water and the eggs permitted to develop for 48 hours with no supplemental feeding.

After 48 hours the veliger larvae, fully protected by their shells, were collected by passing the contents of each culture jar through a 325-mesh stainless steel screen. The screen retained the shelled veligers but permitted undeveloped eggs, and embryos that had not progressed to the shelled stage, to pass through. The normal larvae from all culture jars were thus collected and pooled in 4 to 7 liters of sea water in a tall narrow jar. After thoroughly mixing the contents of this jar with a perforated plastic plunger, to obtain a uniform distribution of the larvae, a sample was withdrawn and the number of larvae in one ml. of the pooled culture determined. By keeping the pooled culture thoroughly mixed, with the larvae uniformly suspended, and taking appropriate volumes, the desired number of larvae could be introduced into each culture. The larvae of all cultures were, therefore, closely comparable in size and quality, as well as number, when the different food treatments were started on the second day.

All culture jars were kept covered so they were uniformly dark and all were in a common water bath. Although the temperature of the water bath fluctuated between 21.0° and 23.0° C. all cultures were equally affected. The sea water in all cultures was replaced with fresh cotton-filtered sea water every second day (Loosanoff and Davis, 1950).

Neither aeration nor mechanical agitation is necessary for normal development of oyster larvae when the water is changed every second day. We did not, therefore, use either aeration or mechanical agitation in these experiments as it is difficult to insure equality of such treatments in a series of cultures.

Samples were taken whenever desired by first washing the entire contents of a culture jar onto the 325-mesh screen, then re-suspending the larvae in 1000 ml. of sea water in a parallel-sided graduated cylinder. To insure uniform distribution of the larvae, the contents of the cylinder were thoroughly mixed with a per-

forated plastic plunger and a one-, two- or five-cc. sample was withdrawn and preserved. All the larvae in a sample were transferred to a Sedgwick-Rafter cell and examined under a compound microscope. Larvae that were living at the time the sample was taken could thus be identified and counted to determine the per cent surviving and a random sample of 100 of these larvae was measured to obtain growth data.

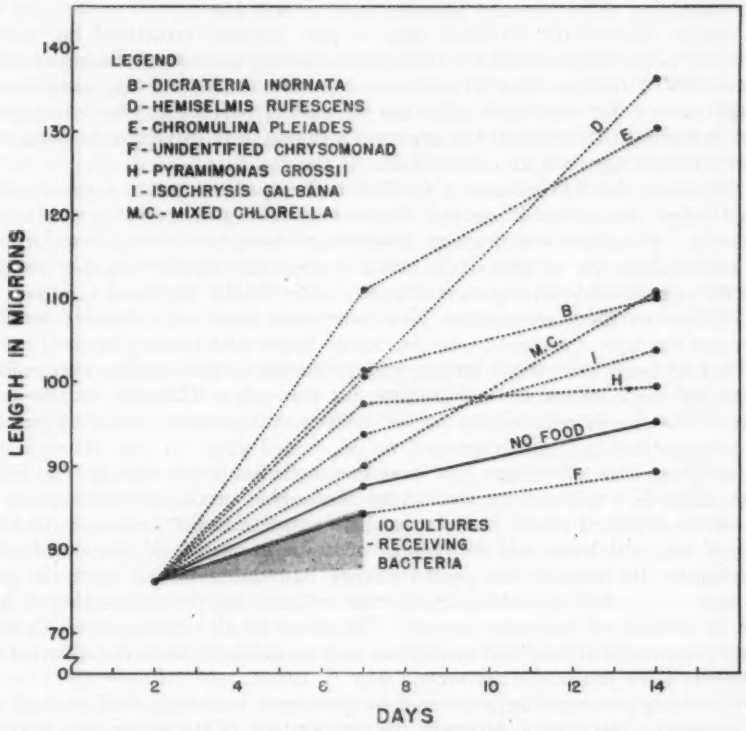

FIGURE 1. Growth of larvae of *C. virginica* fed different foods. Each point on a curve represents the mean length of 100 larvae. Flagellates D, E, and I were fed at the rate of 10,000 per ml./day. Flagellates H and F were fed at the rate of 5000 per ml./day. Flagellate B was fed at the rate of 10,000 per ml./day for first seven days and 5000 per ml./day thereafter.

Eighteen parallel cultures were used in the first of the present series of experiments. Each of the first nine received a different species of the marine bacteria isolated by Dr. Burkholder, six of the remaining each received a different species of the marine flagellates received from England, one received *B. coli* plus a bacteriophage, one received our "mixed Chlorella" and the final culture, which received no supplemental food, served as the control.

The larvae in all ten cultures receiving bacteria and those in the culture receiving flagellate F, an unidentified chrysomonad, grew less rapidly than did those in the unfed control culture (Fig. 1). Moreover, in the ten cultures receiving bacteria, the larvae were all dead by the eleventh day. We can assume that these bacteria and flagellate F were not utilized.

In the remaining six cultures that received supplemental food, the larvae grew more rapidly than did those in the unfed control. We can conclude, therefore,

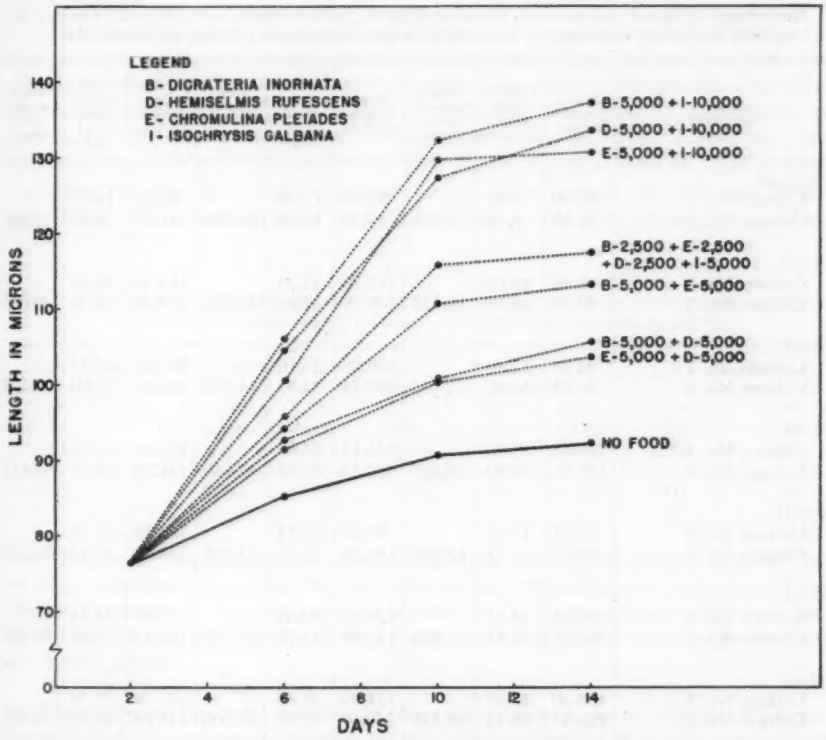

FIGURE 2. Growth of larvae of *C. virginica* fed combinations of flagellates. Each point on a curve represents the mean length of 100 larvae from each of duplicate cultures. Numbers following letter designation of flagellates indicate rate of feeding in number of flagellates per ml./day.

that oyster larvae probably do utilize the five species of flagellates, *Dicrateria inornata, Chromulina pleiades, Hemiselmis rufescens, Isochrysis galbana* and *Pyramimonas grossii,* as well as our "mixed Chlorella."

Unfortunately, due to difficulties in mass production of the flagellates, we were not able to feed equal numbers of each flagellate nor were we able to keep the rate of feeding constant in the larval culture receiving flagellate B (Dicrateria).

In this culture Dicrateria was added at the rate of 10,000 cells per ml./day for the first seven days but after that it was necessary to reduce the rate to 5000 cells per ml./day.

Flagellate I (Isochrysis) and flagellate D (Hemiselmis) were added at the rate of 10,000 cells per ml./day throughout the experiment, while flagellates F (the unidentified chrysomonad) and H (Pyramimonas) were added at the rate

TABLE I

Mean length of larvae (in microns), receiving different food treatments, at 6, 10 and 14 days with growth increments calculated as mean length minus mean length of 2-day-old larvae (76.5μ)

Food treatment	Mean length 6 days	Growth increment 2–6 days	Mean growth increment	Mean length 10 days	Growth increment 2–10 days	Mean growth increment	Mean length 14 days	Growth increment 2–14 days	Mean growth increment
No food									
Culture No. 1	85.90	9.40		92.40	15.90		93.25	16.75	
Culture No. 2	84.60	8.10	8.750	89.30	12.80	14.350	91.35	14.85	15.800
B+E									
Culture No. 1	95.60	19.10		112.20	35.70		113.35	36.85	
Culture No. 2	92.75	16.75	17.925	109.35	32.85	34.275	113.30	36.80	36.825
B+D									
Culture No. 1	93.80	17.30		100.60	24.10		102.83	26.35	
Culture No. 2	91.85	15.35	16.325	100.75	24.25	24.175	108.50	32.00	29.175
B+I									
Culture No. 1	106.65	30.15		133.75	57.25		139.60	63.10	
Culture No. 2	105.40	28.90	29.525	130.55	54.05	55.650	134.75	58.25	60.675
E+D									
Culture No. 1	91.95	15.45		99.25	22.75		103.70	27.20	
Culture No. 2	90.85	14.35	14.900	101.70	25.20	23.975	103.95	27.45	27.325
E+I									
Culture No. 1	98.65	22.15		127.10	50.60		129.95	53.45	
Culture No. 2	102.35	25.85	24.000	132.25	55.75	53.175	131.35	54.85	54.150
D+I									
Culture No. 1	104.30	27.80		125.00	48.50		131.20	54.70	
Culture No. 2	104.95	28.45	28.125	129.40	52.90	50.700	135.75	59.25	56.975
B+E+D+I									
Culture No. 1	94.75	18.25		116.05	39.55		116.90	40.40	
Culture No. 2	97.05	20.55	19.400	114.70	38.20	38.875	117.75	41.25	40.825

of 5000 cells per ml./day. Moreover, since this was a preliminary experiment, single cultures were used and the results are consequently less reliable than in later experiments where duplicate or triplicate cultures were used to test each food. Isochrysis, for example, appears to be a comparatively much poorer food here than it proved to be in later experiments. Note that the larvae fed "mixed Chlorella" and those fed flagellate D (Hemiselmis) grew slightly faster after the

seventh day, while those receiving other foods grew somewhat less rapidly the second week (Fig. 1). This may indicate that these two forms are more readily utilized by the larvae in the later stages of development.

Subsequently, in experiments of factorial design, we have fed oyster larvae various combinations of those flagellates which the previous experiment had shown the larvae could utilize. We hoped to estimate the relative value of the different flagellates as foods for oyster larvae, and to determine whether some combination of them might provide a more balanced diet which would result in more rapid larval growth.

The results of a typical experiment of this kind show that certain combinations did appear to give more rapid growth of larvae than others (Fig. 2). The combination of the flagellates B + E, for example, gave more rapid growth than either the combination B + D or E + D. Nevertheless, the greatest differences are correlated with the total number of flagellates given per day. Thus, the three combinations B + I, D + I, or E + I, each consisting of a total of 15,000 flagellates per ml./day gave definitely more rapid growth than did the combination B + E + D + I which consisted of a total of only 12,500 cells per ml./day. More-

TABLE II

Ratios of the mean growth increments, due to each food combination, to the mean growth increments of larvae in the unfed control cultures. (Standard error of averages 3.75%)

Days	No food	B+E	B+D	B+I	E+D	E+I	D+I	B+E+D+I
2–6	1	2.049	1.866	3.374	1.703	2.743	3.214	2.217
2–10	1	2.389	1.685	3.878	1.671	3.706	3.533	2.709
2–14	1	2.331	1.847	3.840	1.729	3.427	3.606	2.584
Average	1	2.256	1.799	3.697	1.701	3.292	3.451	2.503

over, this latter combination in turn gave slightly more rapid growth than did any of those combinations consisting of only 10,000 flagellates per ml./day. Thus, the total number of flagellates added appears to be an important factor and suggests that the different species of flagellates may be of nearly equal value as foods for oyster larvae.

Statistical tests showed that the differences in rate of larval growth, attributable to differences in the total number of flagellates present, were indeed significant. Nevertheless, to account completely for the results observed it is also necessary to assume that the different species of flagellates were not of equal value as foods for oyster larvae.

An analysis of variance of the growth increments (Table I) showed that the average difference between growth increments of duplicate cultures was not significant. We are justified, therefore, in using the ratio of the mean growth increments of the duplicate cultures as a relative measure of the efficiency of the different foods. Dividing the mean growth increments for each period by the mean growth increment of the unfed cultures for the same period gives relatively constant ratios (Table II). An analysis of variance showed that differences between the ratios for the 2–6-, 2–10- and 2–14-day periods were not significant. The average ratio

can be used, therefore, as a numerical estimate of the relative over-all efficiency of the different food combinations at the particular concentrations used in this experiment.

This suggests that if the effect on growth of larvae on any given food really is constant, the uncontrolled factors responsible for the growth of unfed cultures have a multiplicative power on the effect of the different food combinations. If we postulate, in addition, that the effect of each species of flagellate is proportional to its concentration and independent of the presence or absence of other foods, we can formulate the following equation for the growth increment (Y) of any combination of foods: $Y = k\ (1 + b + e + \ldots)$, where k is the uncontrolled variable responsible for the growth of unfed cultures and b, e, . . . are the effects of the foods B, E, etc. Thus, if b is the effect of 2500 *B* (Dicrateria) per cc., e is the effect of 2500 E (Chromulina) per cc., d is the effect of 2500 D (Hemiselmis) per cc. and i is the effect of 5000 I (Isochrysis) per cc., the equation for the growth increment of the food combination $B + E + D + I$ becomes $Y = k\ (1 + b + e + d + i)$, while for the combination $B + E$, in this experiment it becomes $Y = k\ (1 + 2b + 2e)$ and for the unfed cultures is $Y = k\ (1 + 0)$ or $Y = k$. From the design of the experiment and having a value for k (the observed growth increment of unfed cultures) we can calculate values for b, e, d and i using the values given for the food combinations in the average ratio (Table II). Thus b, the effect of 2500 Dicrateria per cc., becomes: $b = 1/7\{[(2.256–1) + (1.799–1) + (3.697–1) + (2.503–1)] - 3/4[(1.701–1) + (3.292–1) + (3.451–1)]\}$ or $b = 1/7(6.255–4.083) = 0.3103$.

Similarly, the separate effects of the other flagellates become $e = 0.185$, $d = 0.110$, and $i = 0.982$, but this ratio compares 2500 of each B, E and D with 5000 of I. When all are adjusted to the 10,000 level so that we compare the flagellates cell for cell, they become $b = 1.240$, $e = 0.740$, $d = 0.440$ and $i = 1.964$. Since these values were taken from the average ratio of the food combinations, the values represent the over-all efficiency of the foods throughout the experiment.

Similar ratios or estimates of food values can be worked out for the separate four-day periods; corrected by successive approximations and adjusted to the 10,000 level for each flagellate they are:

	b	e	d	i
2–6 days	1.372	0.788	0.776	1.716
6–10 days	1.616	1.112	−0.820	3.226
10–14 days	3.776	−2.504	5.123	0.450

The value of most of the flagellates varies considerably. These variations probably represent day-to-day fluctuations in both physiological state and purity of the flagellate cultures used as foods, for it is known that in many micro-organisms the chemical composition varies with their physiological condition. Thus, although B (Dicrateria), in this experiment, appears to be a better food than E (Chromulina) in approximately 50 per cent of the experiments of this series the reverse is true. It seems probable, therefore, that differences in rate of growth of oyster larvae brought about by variations in the physiological condition of Chromulina and Dicrateria are as great or greater than differences dependent upon which of these two species the oyster larvae are fed.

Flagellate D (Hemiselmis) appears to be the poorest of these four species. However, the high value for the 10–14-day period may indicate, as in the previous experiment, that Hemiselmis is more readily utilized by older larvae. Unfortunately, this flagellate culture was lost and we could not obtain further data on its effect on the rate of growth of oyster larvae.

Bruce, Knight and Parke (1940), in feeding experiments on larvae of the European oyster, *Ostrea edulis,* rated flagellate I as a "good to very good" food while rating flagellate B as "fair." Although their tabulated data seem to indicate that flagellate F was utilized and that flagellate D was not, the authors do not discuss these two flagellates further.

The second part of the problem was to determine whether, by combining the various flagellates, we could provide a diet that would bring about more rapid growth of oyster larvae than could be obtained by feeding an equivalent number of cells of a single species. From a statistical viewpoint, if the separate growth increments due to the different foods, as calculated above, can be combined ac-

TABLE III

Differences between observed and calculated mean lengths of oyster larvae receiving different foods

Foods	6 days			10 days			14 days		
	Observed	Calculated	Diff.	Observed	Calculated	Diff.	Observed	Calculated	Diff.
No food	85.2	84.8	−0.4	90.9	90.9	0.0	92.3	92.3	0.0
B+E	94.2	93.7	−0.5	110.2	110.3	+0.1	113.3	113.2	−0.1
B+D	92.8	93.7	+0.9	100.7	100.7	0.0	105.7	108.8	+3.1
B+I	106.0	104.7	−1.3	132.2	134.3	+2.1	137.2	137.1	−0.1
E+D	91.4	91.3	−0.1	100.5	100.6	+0.1	103.8	103.9	+0.1
E+I	100.5	102.3	+1.8	129.7	130.2	+0.5	130.7	130.0	−0.7
D+I	104.6	102.2	−2.4	127.2	126.1	−1.1	133.5	133.2	−0.3
B+E+D+I	95.9	98.0	+2.1	115.4	114.7	−0.7	117.3	119.6	+2.3

cording to the formula to give a satisfactory fit to the growth increment observed when these foods are used in combination, we will have no reason to believe that there are interactions. In other words, if the effect of B and the effect of E can be added together as in the formula to give a growth increment that agrees closely with that observed when B and E were fed in combination, we will have no reason to believe that the combination B + E is any better as a food than equivalent quantities of B or E alone.

A statistical analysis of the results of the previous experiment shows no evidence that any of the combinations of flagellates tested results in more rapid growth of oyster larvae than would equivalent amounts of any of the flagellates separately. The observed mean lengths of larvae, receiving combinations of the different flagellates as foods, were compared with the mean length calculated from the values of the separate foods given above as the sum of the growth increment given by the formula plus the mean length of larvae in the unfed control cultures (Table III). At six days the maximum difference between observed and calculated mean length is 2.4 μ, at 10 days it is 2.1 μ, and at 14 days it is 3.1 μ. These differences are of

the same order of magnitude as differences between parallel cultures receiving the same treatment and it can be shown that the fit between calculated and observed mean lengths is satisfactory. Therefore, we have no reason to believe that the foods are not completely additive, and no interaction is indicated. Bruce, Knight and Parke (1940) believed that a combination of the flagellates H and I

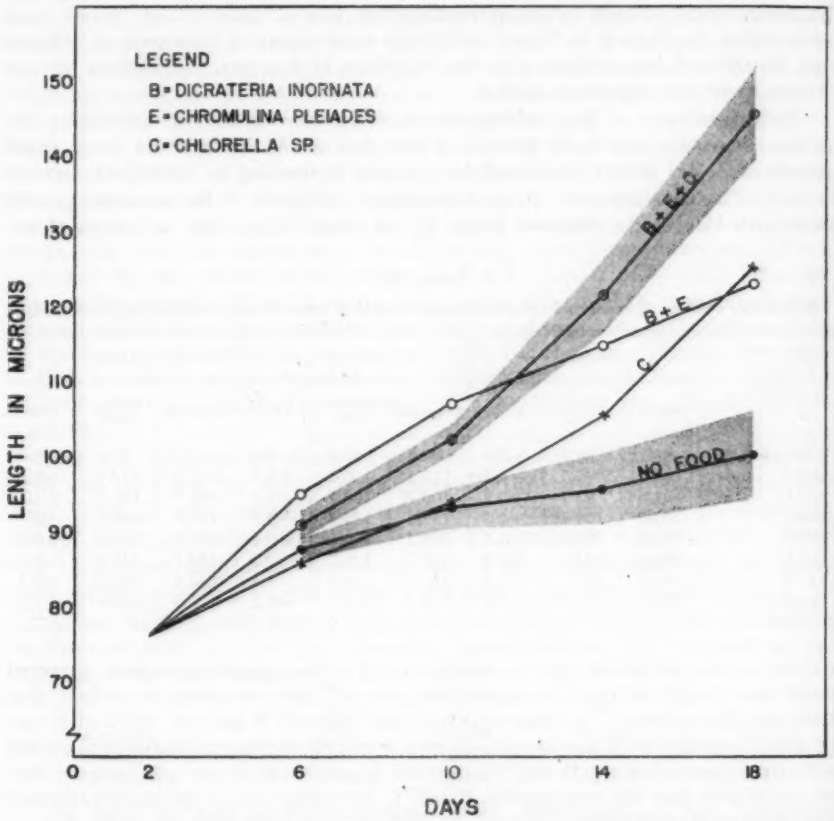

FIGURE 3. Growth of larvae of *C. virginica* fed *Chlorella* sp. alone and in combination with flagellates. Each point on a curve represents the mean length of 100 larvae from each of triplicate cultures. Shaded areas represent 95 per cent confidence bands. Flagellates B and E fed at the rate of 5000 per ml./day and *Chlorella* sp. fed at the rate of 50,000 per ml./day.

was especially suitable as food for larvae of *O. edulis* but did not test for an interaction. Thus far we have not tested this combination with larvae of *C. virginica.*

Studies to determine what concentration of any single species of these flagellates would be required to induce the maximum rate of growth of oyster larvae are still

in progress. Preliminary tests indicate, however, with Dicrateria alone as the supplementary food, that this flagellate must be fed at the rate of 25,000 cells per ml./day (the highest rate tested), or higher, to produce the most rapid growth of larvae possible in cultures such as ours, which contain approximately 5000 larvae per liter. Isochrysis has not been tested in varying concentrations, but the larvae grew rapidly when this flagellate was fed at the rate of 20,000 cells per ml./day. When Chromulina was used as the supplemental food, however, the larvae grew slightly faster in cultures receiving 15,000 cells per ml./day than in cultures receiving 20,000 cells per ml./day.

Our experiments indicate, therefore, that while the concentration of flagellates required to give the most rapid growth of larvae probably varies with the species of flagellate, none of the flagellates that were utilizable gave toxic effects when added to our larval cultures at rates up to 15,000 cells per ml./day and for certain species up to 25,000 cells per ml./day. Korringa (1949), on the other hand, although he obtained good growth of larvae and a heavy spatfall of *O. edulis* in his 1947 experiments with concentrations of flagellates between 10,000 and 20,000 per ml., concluded from later experiments that (p. 4) "water initially containing more than 5000 flagellates, or a commensurable great number of other phytoplankton, should be mistrusted as it may contain toxic concentrations of phytoplankton metabolites from the first day the tanks are filled." Imai and Hatanaka (1949) indicate that in culturing larvae of *Crassostrea gigas,* they strive to keep the concentration of the flagellate, *Monas* sp., at a concentration of only 1000 to 2000 per ml. in their cultures. These authors have, at maximum, only about 200 larvae per liter, however, and such low concentrations of flagellates would provide too small a quantity of food to induce appreciable growth of larvae in cultures such as ours with a concentration of approximately 5000 larvae per liter.

In other experiments we sought to determine the effect of the bacteria-free culture of *Chlorella* sp. on the rate of growth of oyster larvae. In these experiments *Chlorella* sp. was tested both alone and in combination with some of the flagellates (Fig. 3). Using triplicate cultures for each treatment, one trio served as a control and received no supplementary food, one trio received *Chlorella* sp. alone (50,000 per ml./day), one trio received a combination of flagellates B + E (5000 per ml./day of each) and one trio received *Chlorella* sp. (50,000 per ml./day) in addition to the combination of flagellates B + E (5000 per ml./day of each).

Our "mixed Chlorella" culture, although apparently being more effectively used by larvae in the later stages of development, had been utilized from the start (Fig. 1). With a pure culture of Chlorella, however, in this and in several repetitions of the experiment, the effect on the growth rate of the larvae during the early stages, although small, is consistently negative (Fig. 3). This is true both when pure Chlorella is added alone, and when it is used in combination with the flagellates. Some time between the sixth and twelfth days, however, the larvae appear to become able to utilize *Chlorella* sp. and it accelerates their growth markedly.

Calculations similar to those previously mentioned give the following values for b, e and c (effect of *Chlorella* sp.):

	b	e	c
2–6 days	0.252	0.329	−0.164
6–10 days	0.191	0.579	0.323
10–14 days	0.598	0.580	2.364
14–18 days	0.620	0.630	3.467

However, when these figures are adjusted to the 10,000 level so that we are comparing food micro-organisms cell for cell, it becomes obvious that Chlorella is inferior to the flagellates as a food on such a basis, thus:

	b	e	c
2–6 days	0.504	0.658	−0.033
6–10 days	0.382	1.158	0.065
10–14 days	1.196	1.160	0.473
14–18 days	1.240	1.260	0.693

Again there is no evidence of an interaction of food organisms and the growth increment of the combination B + E + Chlorella is given by $Y = k(1 + b + e + c)$. These experiments explain our previously published observations (Davis, 1950a) that "mixed Chlorella," while not consistently a good food during earlier larval stages, served quite well as food during later larval stages. The good growth obtained during later larval stages is undoubtedly due to the utilization of Chlorella itself by the oyster larvae. The inconsistency of growth of earlier larval stages is understandable since, from these experiments, we know that during these stages the larvae do not utilize Chlorella. Obviously, then, growth during the early larval stages, when "mixed Chlorella" was used as a food, was due to other forms which might or might not be present at any given time in our mass culture of Chlorella, and to small amounts of food in the sea water, which may, as in the experiment shown in Figure 3, carry the larvae through the early stages until they can utilize Chlorella.

Cole (1936) after a review of the literature on the European oyster (*O. edulis*) states that the conclusion that the larvae are able to develop on Chlorella is not supported by results of well designed critical experiments. Presumably the larvae are unable to digest this alga. We have shown here, however, that Chlorella is utilized by older larvae of *C. virginica* and we have unpublished data showing that larvae of *Ostrea lurida*, a species closely related to *O. edulis*, can be reared to metamorphosis on either mixed Chlorella or a bacteria-free culture of Chlorella.

A number of cultures of *C. gigas* have been reared to metamorphosis (Davis, 1950b) using our "mixed Chlorella" as a food, although in general larvae of this species are similar to larvae of *C. virginica* in being limited as to the types of micro-organisms they can utilize as foods. In addition Loosanoff and Davis (1950), Loosanoff, Miller and Smith (1951), and Loosanoff and Marak (1951) have reared *O. edulis*, *Venus mercenaria*, *Mya arenaria* and several other species of lamellibranch larvae using our "mixed Chlorella" as the chief food. Such results certainly indicate that the ability to utilize Chlorella as a food is common to several species of lamellibranch larvae.

In the course of our experiments certain differences in rate of growth of oyster larvae, in different members of a trio or pair of cultures receiving the same treatment, appeared to be due to differences in the number of larvae present in the culture. An experiment in which triplicate cultures at each of four different con-

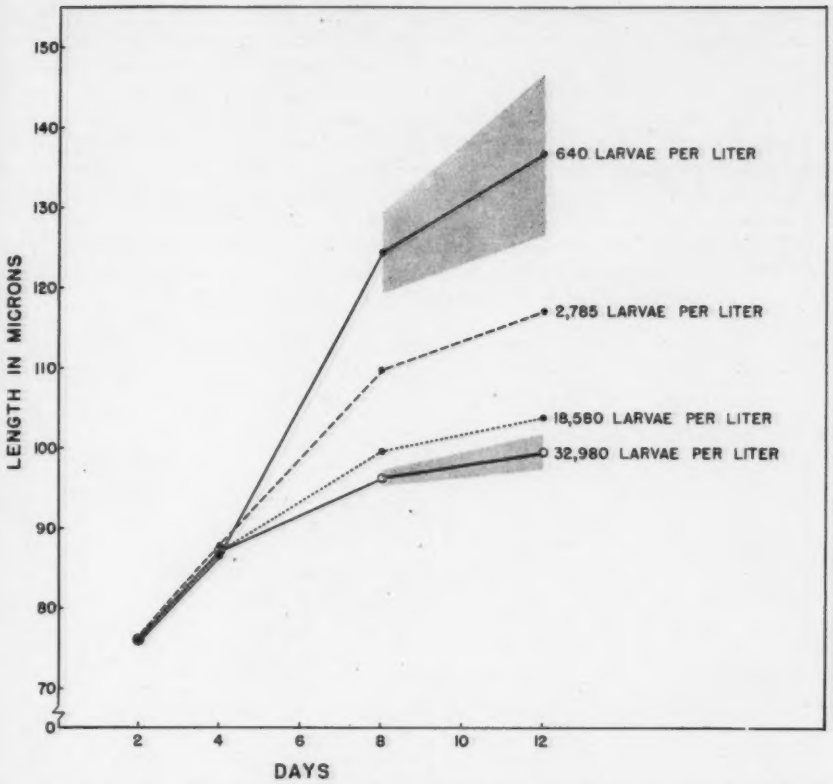

FIGURE 4. Growth of larvae of *C. virginica* cultured at four different concentrations with food constant at 50,000 cells per ml./day. Shaded areas represent 95 per cent confidence bands. Each point on a curve represents the mean length of 100 larvae from each of triplicate cultures.

centrations of larvae were used indicated an inverse relation between the concentration of larvae and their rates of growth (Fig. 4). These cultures all received 50,000 cells per ml./day of our "mixed Chlorella" as a supplementary food.

The between-culture variations, at the two lower concentrations of larvae, were abnormally great, while at the higher concentrations the between-culture variations were normal. Thus at 14 days the average sizes of larvae in the various cultures were as follows:

Number of larvae per liter	Culture number 1	Culture number 2	Culture number 3
640	140.0	140.1	125.17
2,785	110.50	111.57	126.55
18,580	102.65	103.35	105.35
32,980	98.35	98.70	101.60

The 95 per cent confidence bands (Fig. 4) were calculated with the inconsistent values included. We are probably justified in concluding that with all cultures receiving equal quantities of this food, there is an inverse relation between the concentration of larvae in a culture and their rate of growth, at least after the eighth day. The failure of the inverse relation to appear earlier is probably due to the inability of oyster larvae to utilize the "mixed Chlorella" readily during the earlier stages of development.

We do not know whether similar results would be obtained with other foods, but suspect that they would be, nor do we know what results would be obtained if the quantity of food were kept proportional to the concentration of larvae. The results of the experiment suggest, however, that the concentration of larvae in a culture must be considered in comparing rates of larval growth.

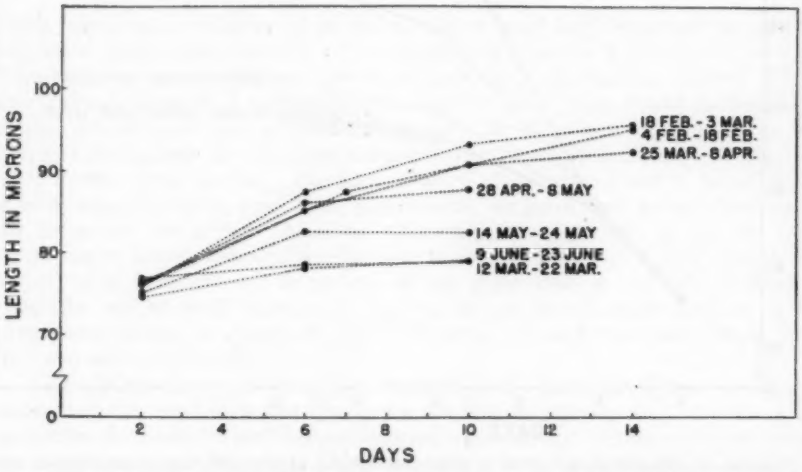

FIGURE 5. Growth curves of unfed control cultures of *C. virginica* larvae showing variations between seven successive experiments. Each point on a curve represents the mean length of 100 larvae from each of duplicate or triplicate cultures.

Other differences, which make comparison of rates of larval growth difficult, are those between successive cultures in which the larvae receive identical treatments. The factor or factors (k) responsible for these differences affect not only the rate of growth of larvae but also, in some experiments, their survival as well. The growth curves of the larvae in the unfed control cultures of seven successive experiments illustrate these differences (Fig. 5). In the 1952 experiments of February 18–March 8 and of February 4–18, the curves appear normal since growth of the larvae in the unfed cultures, although slow, was continuous throughout the 14 days of the experiments. The growth of larvae appears subnormal throughout the May 14–24 experiment, when compared to the above curves, and in the experiments of March 12–22 and June 9–19 almost no growth occurred in these unfed cultures. In the experiment of March 25–April 8 the flattening of

the curve between the tenth and fourteenth days, which reflects an almost complete lack of growth of the larvae during this period, appears abnormal. In the experiments of April 28–May 8 and May 14–24, similar abnormal flattening of the growth curves occurred between the sixth and tenth days.

In each of the three experiments in which the flattening of the growth curves of the unfed cultures occurred after the sixth day, there were also seven pairs of cultures receiving different foods, yet all cultures showed similar flattening of their growth curves simultaneously.

This flattening of growth curves cannot be considered a normal deceleration of growth since it is not correlated with either age of larvae (Fig. 5), or with size (Figs. 2 and 5). Moreover, a pronounced simultaneous upswing of growth curves in all cultures was noted in one experiment and minor simultaneous upward trends were noted in several others. Such simultaneous changes in the growth curves of the larvae in all cultures of an experiment, regardless of food treatment, must be a reflection of variation in some unknown factor or factors common to all cultures. The factors common to all cultures, and least susceptible to control, are the physical, chemical and microbiological constituents of the sea water in which the larvae were reared.

Also strongly suggestive of a variation in the physical or chemical constituents of the sea water was the variation in peak densities of flagellate cultures grown in media prepared from sterilized sea water enriched with constant amounts of nutrient salts. Culture media prepared from sea water taken during periods of poor larval growth gave lower peak densities of flagellates than did media made up from sea water taken during periods of good larval growth.

In all of our experiments, in cultures that received flagellates known to be utilizable, the larvae have grown more rapidly than those in the parallel unfed control cultures. Yet we have not been able to overcome completely the effects of the unknown factor (or factors) by supplemental feeding. For example, under the conditions existing during the May 14–24 and June 9–19 experiments, feeding a combination of the flagellates Isochrysis and Dicrateria produced only about 1/5 as much growth of larvae, in ten days, as did identical concentrations of the same combination of flagellates in the experiment of March 25–April 8. This suggests that one phase of the action of the unknown factor may be to affect the ability of the larvae to utilize the food that is available.

Several authors have suspected unidentified variations in sea water of affecting their results. Loosanoff, Miller and Smith (1951) noted a lack of uniformity of results in consecutive experiments with larvae of *Venus mercenaria* and considered it possible (p. 75) "that at different times the water itself contained certain dissolved substances which, in a manner not yet understood, affected the rate of development of bivalve larvae." Wilson (1951), working in England with polychaete larvae, found a difference between sea water from two different areas and attributed the poor growth of larvae in water from one of these sources to a lack of (p. 18) "some unknown constituent, essential for healthy development" of the species of polychaetes he used. One of these unidentified variants may be that described by Collier, Ray and Magnitzky (1950) who reported a substance in sea water that can be measured photometrically with N-ethyl carbazole, the concentration of which could be correlated with the pumping rate of oysters.

Wangersky (1952) reported that the substance, measured photometrically with N-ethyl carbazole, was a mixture of dehydroascorbic acid and a rhamnoside. He concludes (p. 685) "that the vitamin is present in the sea largely in the form of dehydroascorbic acid." Although we have not yet tried dehydroascorbic acid, we have added ascorbic acid to the sea water in which the larvae were reared. This did not improve the rate of growth of oyster larvae, but merely resulted in a dense growth of bacteria which killed the larvae. Similar exploratory experiments indicate that additions of Vitamin B_{12}, iodine, Mn^{++}, Fe^{+++}, Zn^{++} or PO_4^{---}, likewise, do not improve the rate of growth of oyster larvae.

To verify that deleterious changes in the sea water in our laboratory system were not causing the poor growth and high mortalities of our June cultures, an experiment was designed to compare the laboratory sea water with sea water taken directly from Milford Harbor in enamel buckets. Parallel cultures of oyster larvae were started, two in sea water from each source. In addition a single culture of larvae of *Venus mercenaria* was started in laboratory sea water at the same time. All cultures received approximately 50,000 cells per ml./day of the "mixed Chlorella" as food.

At 14 days all the oyster larvae in sea water from both sources were dead, while the Venus larvae still appeared healthy and were growing normally. At 18 days the Venus larvae had reached setting size and a count revealed that approximately 55 per cent of the larvae that had been counted 12 days earlier while still in the straight hinge stage were still living. Thus, although growth was somewhat slower than average (Loosanoff and Davis, 1950; Loosanoff, Miller and Smith, 1951) the Venus larvae had lived and grown to setting stage, as in several previous experiments (Loosanoff, 1950), under conditions in which the oyster larvae had all died.

In other experiments water from points several miles offshore in Long Island Sound was used but again no significant difference in rate of growth of oyster larvae was noted. This experiment indicated that the "water factor" that resulted in poor larval growth was not just a local condition. We agree, therefore, with Wilson (1951), who concluded (p. 18) "it is evident that many animals find no difficulty in living and reproducing under water conditions that seem to affect some other species adversely."

We do not yet know what this "water condition" is, nor whether it is the presence of some inhibitory or toxic substance that causes poor growth, or, as Wilson (1951) believes, it is the lack of something necessary for good growth. Our experiments do indicate that one phase of its action is to affect the ability of larvae of *C. virginica* to utilize the food that is present.

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tion of this paper. Thanks are also due my co-workers, D. W. Calhoun, who gave valuable assistance in the statistical analysis of the data, and to W. S. Miller and C. A. Nomejko for assistance in other phases of the work.

Summary

1. None of the 13 species of marine bacteria tested to date was utilized as food by oyster larvae.

2. Five species of flagellates, *Dicrateria inornata, Chromulina pleiades, Isochrysis galbana, Hemiselmis rufescens* and *Pyramimonas grossii,* were utilized as food by oyster larvae, while another, an unclassified chrysomonad, in addition to the three flagellates previously reported, was not.

3. *Chlorella* sp. was not utilized as food by young oyster larvae but was utilized during later larval stages.

4. None of the combinations of foods tried gave any evidence of providing a more balanced diet or more rapid larval growth than could be obtained by feeding equivalent quantities of a single food. The effects of all foods tested, including Chlorella, are additive.

5. When equal numbers of cells are fed, different species of flagellates induce different rates of growth of oyster larvae.

6. Species of flagellates also differ in the number of cells needed to induce the maximum rate of growth of oyster larvae.

7. The maximum concentration of food organisms that can be created in water containing oyster larvae without unfavorably affecting the larvae varies with the species.

8. With the number of food organisms equal, the rate of growth of oyster larvae had an inverse relation to the number of larvae per unit volume.

9. Variations between rates of growth of larvae in cultures receiving the same treatment in successive experiments appear to be due to some variable factor in the sea water that affects the ability of the larvae to utilize the food that is present.

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THE EFFECT OF X-IRRADIATION ON NUCLEASE ACTIVITY AND RESPIRATION OF TETRAHYMENA GELEII W [1]

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The marked resistance of unicellular organisms to the action of ionizing radiations, as opposed to the low resistance of mammals, has been known for some time. However, little information is available concerning the response of specific biochemical processes of single cell animals to irradiation. The process which has probably been most extensively investigated is cell respiration. Barron, Gasvoda and Flood (1949a) summarized the early papers in this field and showed that the respiration of dilute suspensions of sea urchin sperm could be inhibited from 10–66 per cent with from 100–20,000 r. The inhibition was believed to be due to the action of stable organic peroxides produced by x-irradiation of sea water (Barron *et al.*, 1949b). They also found that the utilization of succinate and acetate was impaired in x-irradiated sperm. Billen, Stapleton and Hollaender (1952) observed that a dose of 60,000 r, while decreasing the number of viable cells of a suspension of *E. coli* B/r by more than 99 per cent, had no effect on the initial respiratory rate but decreased the oxygen consumption afterwards. The presence of pyruvate or succinate was more effective than glucose in prolonging the initial period of normal activity.

As part of a general program involving the effects of x-irradiation on enzyme systems of various animals, and specifically, in an effort to extend the knowledge of biochemical changes in irradiated unicellular organisms, the enzymes ribonuclease (RNase) and desoxyribonuclease (DNase) and respiration were selected for the present study. Data obtained by Roth, Eichel *et al.* (1953) suggested that the RNase of rat liver was a highly labile enzyme under conditions of whole body irradiation.

Materials and Methods

Cultures. Pure stock cultures of *Tetrahymena geleii* W were maintained at room temperature in 50-ml. Erlenmeyer flasks containing 10–15 ml. of 2 per cent Difco proteose-peptone plus 0.2 per cent Difco yeast extract. Transfers were made each day from a 48-hour culture using a 2 mm. platinum loop. Cultures for experimental use were prepared daily by inoculating one ml. of the 48-hour stock cultures into a two-liter Erlenmeyer flask containing 750 ml. of medium.

Preparation of cells, homogenates and dry weight determinations. For use, the Tetrahymena from 750 ml. of 48-hour cultures were separated according to the method of Seaman (1949) using 50-ml. conical centrifuge tubes. Our cells,

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however, were concentrated in a volume of 25 ml. Homogenates were prepared by diluting an aliquot of the concentrate (usually 5 ml.) with an equal volume of distilled water and then grinding with silica in an all-glass homogenizer at 0–2° C. until microscopic examination revealed no unruptured cells. The homogenates were centrifuged at 5000 r.p.m. in an International No. 1 centrifuge for five minutes to remove the silica and cell fragments. Dry weight determinations were made in triplicate at 80° C. (Seaman, 1951) on the whole cells or homogenates. The dry weights of one ml. of the whole cell concentrates and one ml. of the 1:2 homogenates averaged 9.16 (6.57–13.15) and 3.68 mg. (3.00–4.04), respectively.

Irradiation procedure. Four ml. of the concentrated suspensions or homogenates were added to plastic containers ($7/8 \times 7/8 \times 11/16$ inches) which were then packed in ice in plastic Petri dishes to be irradiated or used as controls. The radiation factors have been described (Wichterman, 1948). The radiation flux was 6300 r per minute. When samples were irradiated at 300,000 or 500,000 r, the ice was renewed every 25 minutes.

Respiration studies. Oxygen consumption by the whole cells was measured at 27° C. using standard Warburg manometry. Each vessel contained one ml. of 0.1 *M* Na_2HPO_4–KH_2PO_4 buffer, pH 7.4, one ml. of cell concentrate and 0.8 ml. of distilled water in the main compartment and 0.2 ml. of 10 per cent NaOH in the center well. The vessels were allowed to equilibrate 10 minutes, the stopcocks closed and readings taken at either 10- or 15-minute intervals for one hour. The vessels were shaken at a rate of 120 oscillations per minute and the gas phase was air. Generally, 20–30 minutes elapsed between the completion of irradiation and the first reading. The average QO_2 value of normal cells obtained by assay of 18 different cultures was 25.9 (16.5–34.1), while the average oxygen consumption per million cells was 109 mm.^{3} (80–150).

Nuclease determinations. RNase and DNase in homogenates of Tetrahymena were assayed at 25° C. by a modification of the turbidimetric method of McCarty (1948). For the RNase tests, the system consisted of 10 ml. of 0.2 per cent sodium ribonucleate (Nutritional Biochemicals), 10 ml. of veronal-acetate buffer, pH 5.85, and a suitable volume of 1:2 homogenate (usually 2 ml.) added at zero time. In every case, the final volume was made up to 25 ml. with distilled water. The components of the DNase system were 10 ml. of 0.2 per cent sodium desoxyribonucleate containing $MgSO_4$ to give a final Mg^{++} ion concentration of 0.003 *M* in 25 ml., 10 ml. of veronal-acetate buffer, pH 5.20, and 5 ml. of a 1:2 hemogenate. Four-ml. samples of each were withdrawn at various intervals (usually 2, 10, 20, 30, 40 and 60 minutes), added to 4 ml. of 1 *M* HCl in 18×150 mm. test tubes and the turbidity read after one minute in a Lumetron photoelectric colorimeter (Model 401) at 420 mμ against a distilled water blank. Under the conditions of the test, the RNase reaction rate was generally linear for 40–60 minutes while the DNase exhibited linearity during the entire 60 minutes. For each enzyme the pH employed is based on preliminary observations only, and does not unequivocally represent the pH of optimum activity. One unit of enzyme activity is defined as that amount of enzyme which, in 25 ml. of test solution, causes a decrease in optical density of 0.1 in 60 minutes. The specific activity is equal to the number of units per mg. dry weight.

Experimental Results

Respiration studies. The QO_2 values of normal and irradiated cells exposed to 300,000 and 500,000 r are summarized in Table I. It can be seen that respiration was usually significantly inhibited at 300,000 r, the average of twelve runs being approximately 30 per cent. No explanation can be given for the wide range of inhibition observed. In two cases, the inhibition was less than 10 per cent. In two instances assays were performed on irradiated cells after 24 hours. In one experiment, a 25 per cent increase in respiration of the irradiated cells was observed, and in the second, the increase amounted to 125 per cent. When the radiation level was raised to 500,000 r, the average inhibition was 40 per cent.

Table I

The effect of x-irradiation on the respiration of Tetrahymena at 27° C.

Dose (r)	Time after irradiation (hrs.)	Control QO_2	Irradiated QO_2	Change (per cent)
300,000	0	22.2	20.3	− 9
....	0	23.7	21.9	− 8
....	0	16.5	13.6	−18
....	0	34.0	25.5	−25
....	0	32.9	27.9	−15
....	0	23.8	16.7	−30
....	0	34.1*	22.7*	−33
....	0	27.8	17.6	−36
....	0	24.3	13.1	−46
....	0	30.1	15.7	−48
....	0	30.0	22.4	−25
	24	10.0	14.2	+25
....	0	24.4	12.8	−48
	24	8.0	18.0	+125
500,000	0	23.3	12.4	−47
	2.5	25.4	6.2	−76
....	0			−29**
....	0	28.8	16.0	−44
	8	10.8	2.6	−76

* Data obtained with *T. geleii* strain E.
** Based on $mm.^3$ oxygen consumed.

In two instances when assays were run at 2.5 and 8 hours after exposure, the inhibition rose to 76 per cent.

It is well known that several substances will diminish radiosensitivity by interaction with active radicals produced by ionizing radiations (Patt *et al.*, 1950; Smith *et al.*, 1950; Chapman and Cronkite, 1950; Hollaender, Stapleton and Martin, 1951; Forssberg, 1950). Some of these compounds were tested for their ability to protect Tetrahymena exposed to 300,000 r. For each experiment, 2 ml. of the cell concentrates were diluted with 2 ml. of each of the neutralized test compounds, or with 2 ml. of distilled water, and then immediately irradiated. Control organisms were treated identically. After irradiating, one ml. was removed from each plastic container, added to Warburg flasks as previously described and

TABLE II

The effect of various amino acids, glutathione and pyruvate on the respiration of control Tetrahymena and Tetrahymena exposed to 300,000 r. See text for concentration of supplement

Experiment No.	Supplement	Control QO_2	Irradiated QO_2	Inhibition (per cent)
1	——	27.8	17.6	36
	L-cysteine	34.4	28.3	18
	L-methionine	29.4	19.4	34
2	——	30.0	22.4	25
	L-cysteine	37.0	30.0	19
	Glycine	28.9		
3	——	24.4	12.8	48
	L-cysteine	35.4	28.8	19
	DL-homocysteine	24.6	20.8	15
4	——	24.3	13.1	46
	L-cysteine	34.6	31.8	8
5	——	16.5	13.6	18
	Glutathione	19.0		
	Glycine	16.5		
6	——	23.8	16.7	30
	DL-serine	26.5	20.1	24
	Sodium pyruvate	31.0	28.6	8

the oxygen consumption followed for one hour. The results of these studies are listed in Table II. L-cysteine (8.3×10^{-3} *M*) afforded 54 per cent protection (average of four experiments). In experiment 3 it can be seen that DL-homocysteine was equally effective at the same concentration. DL-serine (16.6×10^{-3} *M*) was only slightly effective while L-methionine (6.7×10^{-3} *M*) gave no pro-

TABLE III

RNase and DNase activity of homogenates prepared from normal and irradiated cells. Figures give specific activity

Dose (r)	RNase		Change (per cent)	DNase		Change (per cent)
	Control	Irradiated		Control	Irradiated	
300,000	0.162	0.147	−10	0.018	0.016	−11
....	0.204	0.204	0	0.018	0.018	0
....	0.099	0.104	+ 5	0.011	0.011	0
500,000	0.172	0.172	0	0.017	0.014	−18
RNase and DNase activity of irradiated homogenates						
500,000	0.127	0.054	−57	0.015	0.007	−52
....	0.144	0.082	−43			
....	0.151	0.078	−48			

tection. Sodium pyruvate (1.0×10^{-2} *M*), which is rapidly oxidized by Tetrahymena (Seaman, 1949), gave marked protection also.

It is to be noted that L-cysteine (final concentration 3.0×10^{-3} *M*) stimulated the respiration of normal cells by approximately 35 per cent, while glutathione in equal concentration increased the oxygen consumption by 15 per cent. It is possible that the tripeptide was hydrolyzed, liberating cysteine which was then oxidized. L-methionine (2.2×10^{-3} *M*) and DL-serine (6.0×10^{-3} *M*) augmented

TABLE IV

The effect of L-cysteine on RNase activity of homogenate irradiated at 500,000 r

Experimental conditions	Specific activity	Inhibition (per cent)
Control	0.144	—
X-irradiated	0.082	44
Control + L-cysteine	0.156	0
X-irradiated + L-cysteine	0.156	0

the respiration of normal cells slightly, while glycine (1.0×10^{-3} *M*) and DL-homocysteine (3.0×10^{-3} *M*) had no effect. No auto-oxidation of either L-cysteine or glutathione was observed under the test conditions.

Nuclease determinations. It is apparent from the data presented in Table III that the activities of both RNase and DNase were little changed in homogenates obtained from whole cells immediately after irradiation at 300,000 and 500,000 r. However, when homogenates were prepared from normal organisms and then

TABLE V

The effect of p-chloromercuribenzoate on RNase activity of homogenates irradiated at 500,000 r
See text for description of experiment

Experimental conditions	Specific activity	Inhibition (per cent)
1. Control	0.151	—
2. X-irradiated	0.078	48
3. Control + p-chloromercuribenzoate	0.118	25
4. X-irradiated + p-chloromercuribenzoate	0.036	76
5. X-irradiated + p-chloromercuribenzoate + L-cysteine	0.078	48

irradiated at 500,000 r, both enzymes were markedly inhibited. Irradiation of normal homogenates at 500,000 r in the presence of 3.6×10^{-2} *M* L-cysteine completely protected the RNase (Table IV).

In the light of the importance placed on the relationship between SH-dependent enzymes and *in vitro* inhibition due to ionizing radiations (Barron *et al.*, 1948–49), it seemed desirable to determine whether the RNase obtained from *T. geleii* W was an SH-dependent enzyme. The homogenate was divided into five 2-ml. por-

tions. Before irradiating with 500,000 r, sample 2 received 2 ml. of distilled water and samples 4 and 5, 2 ml. each of sodium p-chloromercuribenzoate (final concentration 2.6×10^{-5} *M*). Samples 1 and 3 were controls without and with p-chloromercuribenzoate. All samples were adjusted to the same pH. Three ml. of each of these solutions were used in the RNase assay which was standard except that 13.4 mg. of L-cysteine were added to the system prepared from sample 5. From the data presented in Table V, it can be seen that the addition of p-chloromercuribenzoate to unirradiated homogenate inhibited the enzyme by 25 per cent. RNase inhibition due to the presence of the SH-binding agent and the effect of x-irradiation was additive, the sum amounting to 76 per cent. The addition of L-cysteine to a mixture of homogenate and p-chloromercuribenzoate immediately after irradiation reversed the effect of the SH-reactant completely but did not alter the inhibition due to radiation.

Discussion

The question of whether the decreased oxygen consumption exhibited by irradiated Tetrahymena is a reflection of the inhibition of oxidative enzymes, mortality, or both must await further investigation. At 500,000 r, mortality is probably a significant factor. Elliott and Slater (1951) have reported that Tetrahymena were killed at 550,000 r. We have observed repeatedly that immediately after irradiating a solution containing 2×10^6 cells per ml. with 500,000 r, only an occasional organism exhibited motility; within 24 hours more of the cells regained the ability to move. However, in the light of only a 40 per cent reduction in respiration at 500,000 r, these observations may imply that many of the non-motile cells were alive and their respiratory processes inhibited. The protection against respiratory inhibition which was afforded the organisms by irradiating them in the presence of cysteine and homocysteine is another illustration of the ability of thiol compounds to antagonize the effects of ionizing radiations. The protection of RNase against x-ray inactivation by the addition of cysteine to homogenates is probably a similar phenomenon. This finding is in good agreement with the report of Holmes (1950) who showed that x-ray inactivation of crystalline pancreatic RNase in dilute aqueous solution could be prevented by glutathione. The protective action exerted by sodium pyruvate against respiratory inhibition is interesting in view of the finding that pyruvate will protect bacterial cells against the lethal action of x-rays and H_2O_2 (Thompson, Mefferd and Wyss, 1951; Hollaender, Stapleton and Burnett, 1951).

The significant difference observed between the *in vitro* and *in vivo* action of x-radiation on the activities of RNase and DNase is also of considerable interest. Since the intact Tetrahymena occupy roughly 5 per cent of the volume of the irradiated solutions, it may be that 95 per cent of the radio-decomposition products formed in the extracellular water are excluded by the cell membranes. Also, the oxygen tension within the cells is presumably lower than in the external water; thus fewer free radicals would be produced *in vivo*. Rupture of the cell membranes by homogenization would expose the cellular enzymes to the action of the entire quantity of injurious products formed.

From the inhibition of RNase by p-chloromercuribenzoate, it would seem that in Tetrahymena this enzyme is SH-dependent. Crystalline pancreatic RNase (calf) has been shown to be inhibited by several SH-compounds (Zittle, 1946).

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Summary

1. The respiration of x-irradiated *Tetrahymena geleii* W decreased 30 per cent at 300,000 r and 40 per cent at 500,000 r. The addition of cysteine to the organisms prior to irradiation at 300,000 r afforded approximately 50 per cent protection as measured by oxygen consumption. Homocysteine and pyruvate also gave marked protection while methionine had no effect. Cysteine and glutathione stimulated the respiration of normal cells appreciably, but methionine, serine, glycine and homocysteine had little or no effect.

2. Ribonuclease and desoxyribonuclease activities were not changed significantly in homogenates obtained from whole cells which were irradiated at 300,000 and 500,000 r. However, both enzymes were inhibited by about 50 per cent in homogenates which were irradiated at 500,000 r. For RNase, this inhibition was completely reversed by irradiating in the presence of cysteine. Inhibition of RNase of normal Tetrahymena homogenate by p-chloromercuribenzoate was reversed by cysteine.

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STUDIES ON THE DISTRIBUTION OF VITAMIN B_T (CARNITINE)

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The effect and isolation of a new vitamin of the B-complex, B_T, which is required by the mealworm and certain other beetle larvae, and its identification as carnitine has previously been reported (Fraenkel *et al.*, 1948; Fraenkel, 1951a, 1951b; Carter *et al.*, 1952). It was stated that B_T had a wide distribution in biological materials, that materials of animal origin were better sources than vegetable matter, and that the muscles of mammals constituted by far the richest known source. Carnitine was originally discovered in commercial meat extracts in which it occurs in amounts of one to two per cent, and was not known to be a general constituent of living systems. Heretofore, the only available method of assay was by isolation. Thus it was impossible to assay for carnitine in substances which contain it in quantities smaller than that found in muscle. With the development of a much more sensitive testing method, using growth and survival of *Tenebrio* as the criterion (Fraenkel, 1951a), it has now become possible to assay for carnitine in a great variety of biological materials.

The dietary need for carnitine has so far been demonstrated only for three beetle larvae, the yellow mealworm, *Tenebrio molitor*, the black mealworm, *Tenebrio obscurus*, and another related species, *Palorus ratzeburgi*. The present paper concerns the levels of carnitine in a great variety of sources, animal and vegetable, and also gives evidence of a synthesis of carnitine in organisms which can develop in the absence of carnitine. Throughout this paper the terms "carnitine" and "B_T" will be used almost synonymously; the possibility exists, however, that carnitine may not be the only naturally occurring substance with B_T activity. We are, therefore, assaying, strictly speaking, for B_T activity and not for carnitine.

Methods of Assay and Tabulation

The methods of assaying and expressing results have been previously described in detail (Fraenkel *et al.*, 1950; 1951a). Briefly, *Tenebrio* larvae grow slowly and begin to die after 3–4 weeks on a "synthetic" diet consisting of 20 parts casein, 80 parts glucose, one part cholesterol, two parts McCollum's salt mixture No. 185, 10 parts water and the following vitamins of the B-complex (expressed as μg./g. of the dry diet): thiamin 25, riboflavin 12.5, nicotinic acid 50, pyridoxin 12.5, pantothenic acid 25, choline chloride 500, inositol 250, folic acid 2.5 and biotin 0.25. When a good source of B_T or 0.35 μg./g. carnitine is added to the "basic" diet, the larvae grow at a steady rate and survive.

Table I shows the result of an experiment with graded doses of carnitine. It can be seen that survival is optimal at levels of 0.375 μg./g., while the weights still increase at levels of 0.75 and 1.50 μg. Growth and survival at the 50 μg. level are virtually identical with that at 1.5 μg. In all our determinations we have fol-

lowed a procedure whereby, in a series of graded doses, that diet which allows optimal survival and good, but not necessarily optimal growth, is assumed to contain 0.35 μg. carnitine/g. dry diet.

With levels of 0.35 μg. carnitine per gram, larvae reach an average weight of about 60 mg. after 9–10 weeks of growth at 29–30 ° C. and 60% relative humidity This level of carnitine is sufficient to maintain normal growth only up to this point. Subsequently, growth gradually slows down and the larvae may develop signs of a B_T deficiency. In order to reach a normal weight of 140 mg., after a period of 13 weeks, and to pupate and emerge as normal adults, a level of 1.5 μg. carnitine per gm. diet is required (unpublished data). To base assays on optimal growth and development to the adult stage, experiments would have to be continued for 25 weeks and would also require two to three times as much food. In order to economize in time and food consumption, the assays were all based on the 9 weeks-60 mg. criterion.

TABLE I

Numbers surviving and average weights of Tenebrio larvae on the basic diet and with the addition of yeast or graded doses of carnitine. Twenty larvae were used in each of the control diets and 60 larvae with each concentration of carnitine; at 30° C. and 60% relative humidity

Weeks	Control diets				μg. carnitine/g. of the dry diet									
	No addition		2% yeast		0.19		0.375		0.75		1.5		50	
	Nos.	Mg.	Nos.	Mg.	Nos.	Mg.	Nos.	Mg.	Nos.	Mg.	Nos.	Mg.	Nos.	Mg.
5	9	7.4	17	9.5	45	8.3	48	8.9	48	10.7	50	10.0	50	10.2
6	6	9.7	17	15.0	42	13.3	48	14.9	46	19.3	49	19.1	50	19.4
7	5	12.2	16	23.5	40	21.3	47	22.3	46	29.6	47	29.8	50	29.2
8	4	13.2	15	31.4	38	24.0	47	31.8	46	42.2	47	41.0	48	45.6
9	4	16.2	15	39.7	36	34.6	47	42.5	45	59.5	47	57.6	48	59.2
10	3	24.3	15	50.7	34	47.5	46	56.0	45	64.6	47	72.5	48	74.6

In assaying different materials for a particular substance, it is important to consider the form in which they exist in the diet. Only finely ground powders, such as flour, dried egg or dried milk, can be directly mixed into the diet in the dry state. In most cases, the materials have to be either homogenized in water, with the use of the Waring Blendor, or Potter-Elvehjem homogenizer, or else prepared as extracts. All such materials are then pipetted into the diets in the required amounts and dilutions. To avoid losses in the extraction process, it is always preferable to use homogenates. The Potter grinder homogenizes soft tissues well, and more resistant tissues can often be homogenized with the Waring Blendor. Wherever a tissue did not lend itself to homogenation suitable for pipetting, extracts had to be prepared. In such cases, tissues were first homogenized or ground in a mortar and then extracted with several portions of boiling water for periods of 30 minutes each, and the combined extracts concentrated to the desired volume. In some cases alcohol was added to aqueous extracts to make a concentration of 75 to 80%. The ensuing precipitates were removed by centrifugation and the alcohol evaporated off over a water bath.

In expressing results, a different procedure was followed with powdered materials or whole homogenates, than with extracts. In the former cases, amounts are expressed as percentages of the dry materials, added to the diet. With extracts, levels are often calculated as percentages of the dry, or in rare instances, wet materials from which the extracts were derived.

Experiments

1. *The B_T content of microorganisms (Table II)*

The effect of B_T was originally discovered in experiments with *Tenebrio* larvae which failed to grow on synthetic diets, but which grew well after two per cent yeast had been added. The B_T content of yeast seems to vary in different preparations. Two per cent dry brewers yeast in the diet, the level routinely used in our

Table II

The B_T content of several microorganisms and materials of vegetable origin

	Minimum amount to give full activity	Maximum amounts tested and found inactive	B_T μg./g. solids	Description of preparation
Microorganisms:				
Escherichia coli I[1]		5%	<1*	Grown on synthetic medium
Escherichia coli II[1]		5%	<1*	Grown on synthetic medium
Escherichia coli III[2]		5%	<1*	Grown on synthetic medium
Streptococcus hemolyticus[3]	1.75%		28	Medium not entirely synthetic
Torula yeast	1–2%		17.5–35	
Brewers yeast I	2–4%		8.8–17.5	Anheuser-Busch
Brewers yeast II	1%		35	Anheuser-Busch
Brewers yeast III	1%		35	Anheuser-Busch
Brewers yeast IV	1–2%		17.5–35	Nutritional Biochemicals
Neurospora[3]	1.25		28	Grown on synthetic medium
Tetrahymena geleii[4]		5%	<1*	Grown on synthetic medium
Vegetable matter:				
Wheat	2.5–5%		7–14	
Corn		10%	<0.5*	
Alfalfa concentrate[5]	1.8%		20	Spray-dried
Rye juice[5]		1.8%	<2*	Spray-dried
Oats juice[5]		1.8%	<2*	Spray-dried

Prepared by: [1] I. Gunsalus, [2] Sharp & Dohme, [3] E. L. Tatum, [4] G. W. Kidder, [5] Cerophyl Laboratories.

* Possibly none.

control experiments, usually ensures optimal survival, but the larvae may still be somewhat underweight (Table I). The B_T activity of three different strains of brewers yeast, obtained from Anheuser-Busch, varied between 1% and 4%. The B_T content of *Torula* yeast and of *Neurospora* was of the same order as that of brewers yeast.

Two of the microorganisms tested, *Escherichia coli* and *Tetrahymena geleii*, were entirely devoid of B_T activity when included in the diet in levels up to 5%. Full activity at this level would have indicated a carnitine content of 7 μg./g., and

there would have been an indication of activity had the carnitine content been only 1 μg./g. It would be difficult to test for such small quantities, because of the large proportion, 40% and more, of assay material, which would have to be included in the diet. Both these organisms were cultured in synthetic media which in all probability were free of B_T. We may, therefore, conclude that these two organisms neither require nor synthesize carnitine, or synthesize it in amounts below the sensitivity of our assay method. On the other hand, *Streptococcus hemolyticus* from a medium which was not entirely synthetic showed B_T activity at the 1.25% level.

2. The B_T content of vegetable matter (Table II)

It was previously shown (Fraenkel, 1951a) that coconut meal, peanut meal and cotton seed meal failed to show any B_T activity in concentrations up to 3% of the diet. The B_T content of all vegetable matter which has so far been tested was low. Of cereal seeds, wheat showed good activity in concentrations of 2.5 to 5%, but corn was inactive in amounts up to 10%. Of a few samples of juices which had been pressed out of young plants and spray-dried, alfalfa juice was active at a level of 1.8%, while rye and oat juice failed to show any activity at the same level.

3. B_T requirements and B_T contents of insects

A dietary requirement for carnitine has so far been demonstrated for three insect species only, *Tenebrio molitor, Tenebrio obscurus* and *Palorus ratzeburgi.* The larvae of at least 15 other species of insects have been successfully grown on basic diets, similar to that used for *Tenebrio,* in the absence of carnitine. The question then arose, whether carnitine was present in the tissues of insects grown in its absence. It would also be of interest to know to what extent the amount of carnitine present in the tissues of *Tenebrio* larvae corresponded to the levels which had been fed in the diet.

a. Carnitine content of Tenebrio larvae

1. *Larvae grown largely in the absence of carnitine.* These larvae had been grown for the first three weeks on the deficient basic diet, then for 10 days on a normal optimal diet (whole meal plus 5% yeast) and subsequently again on the synthetic deficient diet. They continued to grow for a period of about 7 weeks until they reached a weight of 40–60 mg., then stopped growing and ultimately died. Extracts from these deficient larvae, incorporated in diets in amounts corresponding up to 4% larval bodies, failed to show any B_T activity.

2. *Larvae grown on an optimal diet, consisting of wholemeal flour plus 5% yeast* (Table III). Extracts from these larvae, incorporated in diets in quantities corresponding to 1% of dry larval bodies, gave good growth and almost optimal survival. The result indicated that 2% would have ensured optimal survival.

One gram of wheat flour contains 7 μg. B_T (Table II) and the addition of 5% yeast (containing 17.5 B_T/g.) only insignificantly increases that amount. The growing *Tenebrio* larva eats food in amounts of about 4.5 times its ultimate dry body weight. Thus a nearly full-grown larva, which weighs about 100 mg. (40 mg. dry weight), has consumed 180 mg. of food, containing 1.3 μg. of carnitine. Therefore, 32.5 μg. carnitine are consumed per gram dry weight of larvae. If all this

carnitine were retained in the body of the larva, a diet with 2% dried larvae added as the sole source of carnitine would contain 0.65 μg. carnitine per gram. However, larvae grown on this particular diet only showed optimal activity. Since 0.35 μg. B_T is the minimal amount required for optimal activity, it may be assumed that the diet actually contained only 0.35 μg. carnitine per gram. Thus the actual carnitine content of the dried larvae would be 17.5 μg./g. The growing larvae therefore retained almost half the carnitine which they took up in the food.

3. *Larvae grown on graded levels of carnitine.* In one experiment, extracts from *Tenebrio* larvae, which had been grown on diets containing 0.37, 0.75 and 1.5 μg. B_T per gram, were incorporated in diets and tested in quantities correspond-

TABLE III

The B_T content of two beetle larvae, Dermestes vulpinus and Tenebrio molitor, grown in the presence and absence of B_T. Tested as aqueous extracts in their effect on growth and survival of Tenebrio larvae after 9 weeks

	Concentration in diet corresponding to	Grown in the			
		Presence of B_T		Absence of B_T	
		Nos.	Mg.	Nos.	Mg.
Dermestes vulpinus	0.125%	8	66.1	10	55.2
	0.25%	15	72.2	7	71.2
	0.5%	13	67.7	13	77.6
	1.0%	12	89.6	14	85.7
	Controls: no B_T	3	32.6		
	2% yeast	18	74.6		
Tenebrio molitor	0.125%			2	47.0
	0.25%	6	43.7	1	4.5
	0.5%	6	61.6	0	—
	1.0%	12	53.0	0	—
	Controls: no B_T	0	—		
	2% yeast	18	96.1		

Calculated B_T contents (μg./g. dry insect bodies)
grown in presence of B_T: *Tenebrio* 17.5; *Dermestes* 140
grown in absence of B_T: *Tenebrio* none; *Dermestes* 70–140

ing up to 4% of the diets. None of these diets showed B_T activity. It was calculated that even with 1.5 μg. in the diet, and even if all the carnitine had been retained in the larvae, only 0.27 μg. carnitine per gram could have been present in diets containing 4% of dried larval bodies.

In a subsequent experiment the B_T content was determined of larvae which had been grown on synthetic diets containing 1.5 μg. and 50 μg. carnitine/g. respectively. Extracts from larvae, grown on 1.5 μg. carnitine per gram of food, gave an optimal response in quantities corresponding to 7.5% dry larval bodies; and extracts from larvae grown on 50 μg. carnitine/g. showed optimal activity in quantities corresponding to 0.3 to 0.6% larval bodies. Making use of these figures it is calculated that 7.5% of larval tissue, grown at a level of 1.5 μg. B_T/g., would

have provided 0.5 μg. carnitine/gm. diet, if all the carnitine fed to the larvae had been retained. Correspondingly, 0.3–0.6% of larvae raised on a diet with 50 μg./g. B_T could have provided a maximum of 0.35 to 0.7 μg. B_T/g. Since the diet at the levels stated only showed optimal activity which is assumed to correspond to a level of 0.35 μg. B_T/g., it is obvious that in this experiment almost all the carnitine which the larvae had eaten was retained (Table IV).

The B_T contents of larvae grown on diets containing 1.5 and 50 μg. B_T/g. were calculated as 5 μg. and 56 to 112 μg./g., respectively.

In a further experiment, larvae were raised on whole meal flour plus 5% yeast until they reached a body weight of 20 mg. live weight. One portion of these larvae was tested for B_T, and another transferred to a basic carnitine-free diet and tested when the average weight reached 105 mg. The 20-mg. larvae showed good activity at levels of 1.25–2.5% which correspond to a carnitine content of 14–28 μg./g. This is in good agreement with the results of a similar assay referred to

TABLE IV

Relations between B_T content of the food, the retention of B_T in Tenebrio molitor larvae and the B_T content of larvae grown on these foods

	Nature and B_T content of diets of *Tenebrio* larvae to be assayed for their B_T content		
	Whole meal flour + 5% yeast 7 μg. B_T/g.	Synthetic diet 1.5 μg. B_T/g.	Synthetic diet 50 μg. B_T/g.
Smallest quantity of *Tenebrio* tissue to give optimal B_T effect	2%	7.5%	0.3–0.6%
Amount B_T which would have been present in test diets if all B_T taken up had been retained	0.65 μg./g. diet	0.5 μg./g. diet	0.35–0.70 μg./g.
Amount B_T found by bio-assay	0.35 μg./g. diet	0.35 μg./g diet	0.35 μg./g. diet
Calculated B_T content of larvae	17.5 μg./g. tissue	5 μg./g. tissue	56–112 μg./g. tissue

above (Table IV). The other larvae, after having reached a weight of 105 gm., showed no carnitine activity in levels up to 7.5% of the diet. If all the carnitine which was present in the 20-mg. larvae had been retained, the 105-mg. larvae would have been expected to have 5 times less carnitine per unit weight, and would have shown carnitine activity at levels of 6–12%. The fact that no activity was shown at the 7.5% level indicates some loss of carnitine during growth from 20 to 105 mg.

All these tests with *Tenebrio* indicate clearly:

1. That no synthesis of carnitine takes place in *Tenebrio* larvae, and
2. that growing *Tenebrio* larvae retain 50% or more of the carnitine which they take up with the food.

b. The carnitine content of the larvae of Dermestes vulpinus

The larder beetle, *Dermestes vulpinus*, has been raised successfully on a diet consisting of 80 parts casein, 20 parts fructose, one part cholesterol, two parts

McCollum's salt mixture and the mixture of 8 vitamins, as described above (Fraenkel, 1953). A comparison of the carnitine contents of *Dermestes* grown on this diet in the absence of carnitine, or on a mixture of 95% fish meal and 5% yeast, which contains high amounts of carnitine (Table VIII), showed them to be virtually the same (Table III). In both cases *Dermestes* bodies gave good activity in the diet at levels of 0.25 to 0.5%, which corresponds to a carnitine content of 70 to 140 μg./g. Carnitine is therefore synthesized with great efficiency in the *Dermestes* larva.

c. The carnitine content of the larvae of the blowfly, Phormia regina

The larvae of *Phormia regina*, grown on a diet of raw liver, under nonaseptic conditions, showed full B_T activity when incorporated in the diet at a level of 0.062% which corresponds to the very high content of 560 μg. carnitine/gm. (dry). Larvae grown on a synthetic diet in the absence of carnitine under sterile conditions showed full activity in a *Tenebrio* diet in levels of 2%. This corresponds to a carnitine content of 17.5 μg./g. or about 30 times less than the amount found in larvae grown on liver. Carnitine is therefore also synthesized in the fly larva, but at a lower level than in *Dermestes*.

In another test, the carnitine content of fly larvae which had been grown on liver was followed from the fully grown larva through the pupal stage to the young adult. Fully grown larvae, two-, three-, and four-day old pupae and adults one day after emergence were tested. The carnitine content was the same in all stages, about 560 μg./g. dry weight. Since synthesis of carnitine in the fly larva is relatively slow, we may assume that the bulk of the carnitine, which has accumulated during larval development, is retained in the pupa and later incorporated in the tissues of the adult fly.

In summary we may therefore state that synthesis of carnitine takes place in two insect species (*Dermestes* and *Phormia*) which do not require it in the diet, while *Tenebrio*, which requires it in the diet, shows no signs of synthesis under any conditions.

4. The B_T content of eggs, chick embryos and chicks

The hen's egg had previously been shown to contain surprisingly little B_T. A test with dried egg powder suggested full activity at a level of 6% in the diet (Fraenkel, 1951a). Subsequently several tests with raw homogenized egg showed an even lower activity. Three per cent (on a dry weight basis) was entirely inactive and could hardly have shown optimal activity at less than a 12% level. It was then thought that the negative results obtained with raw egg white might have been caused by a toxic egg-white factor. This assumption proved to be incorrect. Extracts made from boiled egg were only a little more active than the corresponding amount of raw egg. Finally, pure carnitine, at levels of 1.5 μg./g. diet, was added to diets which contained raw or boiled egg in concentrations up to 3 per cent. Growth was uniformly optimal in all tests with added B_T. This showed conclusively that the negative results with raw egg could not have been due to the presence of a toxic factor.

Subsequently, it was found that the hatching chick contained demonstrable amounts of B_T. This suggested the synthesis of B_T in the growing chick embryo.

A test was then designed in which eggs (single comb White Leghorn) were incubated, and the embryos and remaining yolk and white portions analyzed for B_T after periods of 8, 12, 16 and 20 days, and in the one-day old unfed chick. The embryos were dissected out and, after removal of the membranes, homogenized in a Potter-Elvehjem homogenizer (8- and 12-day embryos) or the Waring Blendor (16- and 20-day embryos). The results are shown in Table V. All the embryos showed about the same carnitine activity, with optimal effects at levels of 0.5%. No B_T activity was shown in the remaining white and yolk fractions at levels up to 3%, in the 8-, 12- and 16-day egg, and a low activity, at a level of 2.3%, in the 20-day egg. The B_T activity of the whole one-day chick could not be measured accurately, but appeared to be of the same order as that of the embryos. However, tests with three tissues, brain, liver and muscle, showed B_T activities of an order similar to those encountered in several mammals (Table VI).

TABLE V

The B_T activity of egg and of chick embryo and other egg fractions after varying periods of incubation. Tested as whole homogenized preparations (with the exception of muscle—one-day chick) in their effect on growth and survival of Tenebrio larvae after 9 weeks

Period of incubation	Weight of embryo or chick	Material	Minimum amount to give full activity	Maximum amounts tested found inactive	Calculated B_T contents μg./g. dry tissue
0 days		Whole egg		3%	<3
8 days	0.9 g.	Yolk + white		2.8%	<3
		Embryo	0.35–0.7%		50–100
12 days	4 g.	Yolk + white		2.8%	<3
		Embryo	0.4–0.8%		44–88
16 days	14 g.	Yolk + white		3%	<3
		Embryo	0.5%		70
20 days	31 g.	Yolk + white	2.3%		15
		Embryo	0.4%		88
One-day chick	40 g.	Brain	0.5–1%		35–70
		Muscle extract	0.075%		466
		Liver	0.56%		70

If we assume a water content of the 20-day (31 g.) embryo of 80%, and an optimal B_T activity at the 0.5% level, this embryo would have contained 420 μg. carnitine and the egg less than 52.5 μg., assuming an egg of 50 g. (15 gm. dry weight) and optimal B_T activity with more than 10% egg in the diet. It is evident that nearly all the carnitine in the chick must have arisen from synthesis.

5. The B_T content of mammalian tissues, blood and urine

It has been previously shown that various organs of mammals are rich sources of B_T (Fraenkel, 1951a). These determinations were carried out with fat-free powders. Subsequently it was found that the mammalian skeletal muscle showed three to five times higher B_T activity than the best tissue preparations previously tested. More assays have now been carried out using homogenates and extracts from fresh organs from the dog, rat and rabbit (Table VI). With tissue homogenates, prepared with the aid of the Waring Blendor or Potter-Elvehjem homog-

enizer, the calculations in Table VI are based on the solid content of the homogenates. In the case of extracts, the water content of tissues from which the extracts were prepared was not determined at the time. For these calculations a water content of 70% was assumed, which introduces small errors.

The results obtained from fresh dog tissues were of a similar order as those previously determined from tissue powders (Table VI). Brain and nerve contained relatively small amounts of B_T, and the B_T content of blood, on a dry weight basis, was also low, for an animal tissue. The highest concentrations of B_T were

TABLE VI

B_T (carnitine) content of various mammalian tissues, expressed as μg./g. dry tissues

Organism	Tissue	Minimal conc. for optimal activity %	B_T μg./g. dry tissue	Method
Dog I	Muscle, leg	0.03	1120	Homogenate
	Liver	0.12	280	Homogenate
	Brain	0.4	87	Homogenate
	Pancreas	0.33	105	Homogenate
	Small intestine	0.15	224	Homogenate
	Blood	1.0	35	Whole
Dog II	Muscle, leg (normal)	0.03	1120	Extract
	Muscle, leg (paralyzed)	0.03–0.06	560–1120	Extract
	Heart	0.06	560	Extract
	Bladder	0.06–0.12	280–560	Extract
	Nerve	0.25–0.5	70–140	Homogenate
Dog III	Liver	0.25	140	Homogenate
	Kidney	0.085	412	Homogenate
	Liver	0.5	70	Extract
	Liver, autolysed	0.25–0.5	70–140	Homogenate
	Liver, autolysed	0.5	70	Extract from autolysed liver
Rat	Muscle, leg, laboratory diet	0.03–0.06	560–1120	Homogenate
	Muscle, leg, B_T-free diet	0.05–0.1	350–700	Homogenate
	Liver, laboratory diet	0.17–0.34	100–200	Homogenate
	Liver, B_T-free diet	0.15–0.3	112–224	Homogenate
Rabbit	Muscle, leg	0.048	700	Homogenate
	Liver	0.09	370	Homogenate

again found in muscle, with the skeletal muscle highest, a smooth muscle (bladder) lowest, and the heart muscle intermediate. The B_T content of muscle and liver from rabbit and rat differed only slightly from those of the dog.

From the high concentration of B_T in skeletal muscle it was assumed that B_T may play some important function in the metabolism of muscle. In order to determine whether or not the concentration of B_T in the skeletal muscle was dependent on its functioning, one hind leg of a dog was paralyzed by severing the nerve leading to it, and corresponding portions of muscle from the paralyzed and normal leg were analyzed for their B_T content three weeks later. The carnitine

content of the paralyzed muscle was very slightly, and probably insignificantly, lower than that of the normal muscle (Table VI).

Another comparison was made between the B_T content of liver and muscles from rats fed on a normal laboratory diet, which contained carnitine, and others fed on a synthetic diet, which did not contain it. The results suggested that the carnitine contents of these two tissues were not dependent on the supply in the diet, and that therefore, in all probability, carnitine is synthesized in the rat.

In a further experiment the stability of carnitine to the action of tissue enzymes was investigated. Homogenates of dog liver were subjected to autolysis for three days at 30° C. in the presence of toluene. At the end of the period there was a strong smell of putrefaction in the samples. There was no change in the carnitine content as a result of autolysis and putrefaction.

Table VII contains data concerning the B_T content of urine and blood from human subjects. Here the B_T content is expressed as μg./ml. of urine or blood.

TABLE VII

B_T (carnitine) content of urine and blood from human subjects

Sex and age	B_T μg./ml.	Description
		Urine
♂50	132	24-hour sample
Same	132–264	3 hours after heavy meat meal
Same	28–56	20 hours on diet low in B_T
♀21	56	High protein diet
♂23	56	High protein diet
♀22	14–28	Diet high in vegetable and fruit
♂24	28–56	Diet high in vegetable and fruit
♀21	132	After 3 days of starvation
♂25	132–264	After 3 days of starvation
Students	28–56	Pooled sample from about 50 students
		Blood
	7–14	Pooled, whole blood
	7–14	Pooled, plasma

The B_T content of urine showed fairly large variations which seemed to be dependent on the B_T intake in the diet. The highest figures, 132–264 μg./ml., were found after a heavy meat meal and at the end of a starvation period of three days. This, in the case of starvation, may be due to breakdown of muscle substance and concomitant release of B_T. The lowest levels, 14–28 μg./ml., were obtained from persons who had been on a vegetable diet low in carnitine.

The B_T content of human blood was uniformly low in several determinations (7–14 μg./ml.) and the activity of whole blood and plasma was the same.

6. The B_T activity of various extracts

In the preceding pages, the carnitine contents of diverse biological materials have been stated in terms of μg. per gram dry weight of that particular material. When assaying extracts, it is also of interest to know the carnitine activity in re-

lation to the solid content of the extracts. Table VIII contains data about the B_T activity of various extracts which were used in the course of this work. With commercial preparations, nothing is known about the procedures of preparation, but it is assumed that they were mainly hot water extracts. In our own preparations, the homogenized samples were extracted with two or three portions of water at 100° C. In some cases, the hot water extracts, after concentration in an evaporating dish, were treated with alcohol, to give a concentration of 75 to 80 per cent, and the ensuing precipitates removed.

Table VIII shows that meat extracts were by far the most active preparations, containing carnitine in amounts from one to almost three per cent. Whey and liver extracts, which were used in the isolation of carnitine (Carter *et al.*, 1952), contained 5 to 10 times less carnitine than muscle extracts. Fish extracts were

TABLE VIII

The B_T activity of various extracts. The activity is expressed as µg. solids per gram of the dry diet (A) and as µg. B_T per gram solids in the extracts (B)

	A Minimum amount to give optimum effect µg./g.	B B_T per gram solids µg./gm.	Method of preparation
Beef muscle extract (Wilson)	36–72	4860–9720	Aqueous extract from dried muscle powder
Meat juice (Wilson)	15–30	11,650–23,300	Commercial preparation, probably aqueous extract
Difco beef extract	12–24	14,600–29,200	Commercial preparation, for bacteriological use
Liver extract (Wilson)	125	2800	Aqueous extract, alcohol treated
Heart infusion	125–250	1400–2800	Commercial preparation
Whey extract	360	972	Aqueous extract from dried whey (Borden), alcohol-treated
Fish solubles	500	700	Commercial preparation
Fish meal extract	100	3500	Aqueous extract
Yeast (brewers) extract	2000	175	Aqueous extract, alcohol treated
Vitamin T concentrate	3400	103	Commercial preparation (Pharmazell GMBH)

of a similar order of activity. Brewers yeast, the material on which the B_T effect was originally discovered and which had been used in the early isolation work, proved a very inferior source. A standard preparation of vitamin T (Pharmazell GMBH, Raubling, Obb.) proved to be of the same order as yeast extract. (Vitamin T concentrate has the appearance, smell and taste of yeast extract, and, in all probability, is nothing but a crude yeast extract.)

DISCUSSION

Our investigations concerning distribution and requirements of Vitamin B_T (carnitine) have made it abundantly clear that B_T occurs widely, if not perhaps universally, in all living matter and that animals either require it in the diet or else synthesize it. We have demonstrated the presence of B_T in a great variety of

organisms, ranging from yeast to mammals, but have been unable to find it in a bacterium, *Escherichia coli,* a protozoon, *Tetrahymena geleii,* and in corn seeds. It would be rash to conclude from the available data whether or not it is a necessary constituent of all living matter. Our testing method, using growth and survival of an insect, *Tenebrio molitor,* is not sensitive enough to detect amounts below about one μg./gm. of the dry substance. Furthermore, the possibility must be kept in mind that it might occur in some organisms in a form in which it is not available for *Tenebrio.*

At this point we may ask why we would expect carnitine to occur in all living matter. We have seen that it occurs regularly in animals, and that those organisms which do not require it in the diet synthesize it. This, together with the fact that *Tenebrio* requires it in very small quantities, 0.37 to 0.75 μg./g. of the food, that is to say on the catalytical level, would indicate that it functions in a process of vital importance, at least for yeast, higher plants, insects and vertebrates. Processes of this description are known in the functioning of enzyme systems of the intermediary metabolism and are usually characteristic for all living matter.

On the other hand, it is difficult to reconcile the fact, that B_T is required by *Tenebrio* on the level of some of the most potent B vitamins, biotin and folic acid, but occurs in certain tissues, especially muscle, in quantities of one mg./g. This may suggest that it might be involved in two different functions, one in which it acts on the catalytical level, and one in which vastly larger quantities are involved.

Leclerq (1950) stated that the growth rate of *Tenebrio molitor* was greatly increased by the addition of a preparation of vitamin T (Goetsch, 1947) to the synthetic diet and considers the possibility that vitamin T and B_T could be identical. (The similarity in the designation of these two growth factors is entirely accidental.) We have found only weak B_T activity in a vitamin T preparation which entirely excludes this possibility. It has, however, since been shown that "vitamin T," a crude extract from Torula yeast, contains a multitude of factors (Goetsch, 1951; Wacker *et al.,* 1951).

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Summary

1. The procedure in the bio-assay of B_T (carnitine) with the use of *Tenebrio molitor* as the subject has been described. The minimum requirements of carnitine to give optimal survival and nearly optimal growth of the *Tenebrio* larva are 0.35 μg. per gram of the dry diet. For optimal growth 1.5 μg. per gram are required.

2. Yeast and Neurospora contain about 35 μg. carnitine/g., and wheat 7–14 μg./g. No evidence of the presence of carnitine could be obtained from *Escherichia coli, Tetrahymena geleii* and corn.

3. Synthesis of carnitine takes place in two insect species (*Dermestes* and *Phormia*) which do not require it in the diet, while *Tenebrio*, which requires it in the diet, shows no sign of synthesis under any conditions. The growing *Tenebrio* larva retains more than 50% of the carnitine which it takes up in the diet.

4. Little carnitine was found in the hen's egg. It is synthesized in sizeable quantities in the growing chick embryo.

5. In mammalian tissues, by far the largest quantities of carnitine, about 1000 μg./g. of dry tissue, occur in the skeletal muscle. Corresponding figures for other tissues were: heart 560, bladder 280–560, kidney 412, liver 140–280, small intestine 224, brain 87, nerve 70–140 and blood 35 μg./g. dry tissue.

6. In dog muscle which had been paralyzed for several weeks, the carnitine content was not significantly changed. Liver after autolysis had the same carnitine content as before.

7. The carnitine content of pooled samples of human urine was 28–56 μg./ml. In different individuals levels as low as 14–28 and as high as 132–264 μg./ml. were found. This variation depended on the carnitine content of the diet, with the highest figures after a heavy meat meal or a three-day period of starvation, and the lowest after a vegetable diet. Blood contains fairly uniform levels of 7–14 μg./ml.

8. Different commercial preparations of meat extracts had carnitine contents of one to three per cent (dry weight).

9. Since carnitine occurs universally, or almost universally in biological material, and an organism either synthesizes it, or else requires it as a "vitamin," it is considered to be of vital importance in the metabolism of most or all forms of life.

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BLOOD COAGULATION IN ARTHROPODS. III. REACTIONS OF INSECT HEMOLYMPH TO COAGULATION INHIBITORS OF VERTEBRATE BLOOD[1]

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As shown in previous studies by means of the phase contrast microscope (Grégoire and Florkin, 1950; Grégoire, 1951a), hemolymph coagulation in a number of insects appears to be a continuous process, initiated by alterations taking place rapidly in a category of highly labile hemocytes (coagulocytes). These alterations are followed by the development of islands of coagulation in the surrounding plasma. Various degrees of extension of the process occur in different species of insects, from a general clotting, macroscopically visible, to a limited reaction, consisting of a few microscopic islands of coagulation, centered by these hemocytes and scattered in an apparently fluid hemolymph.

The aim of the present study was to investigate by means of the phase contrast microscope the reactions of the insect hemolymph to substances or physical conditions inhibiting the coagulation process in vertebrates and other invertebrates. Insects, chiefly Orthoptera, characterized by a conspicuous clotting process, were selected as experimental material.

The appearance or the absence of the early alterations taking place in the category of highly labile hemocytes and in the neighboring plasma was found to be an expedient test for the evaluation of the degree of interference with the coagulation process brought about by various compounds and experimental conditions.

Material and methods

The species of insects used for the present investigations were *Locusta viridissima* (great green grasshopper), *Chorthippus* sp. (short-horned grasshopper), *Gryllulus domesticus* (house cricket), *Periplaneta americana* L. (cockroach), *Blabera gigantea* (South American cockroach), *Mantis religiosa* (praying mantis), *Gryllotalpa gryllotalpa* (mole-cricket), *Carausius morosus* (stick insect) (Orthop-

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tera); *Forficula auricularia* (earwig) (Dermaptera); *Nepa cinerea* (water-scorpion), *Lethocerus cordofanus* Mayr (Belostomatidae, Belgian Congo) (Hemiptera); *Meloe proscarabaeus* (oil beetle), *Tithoes frontalis* (Longicorn from Belgian Congo) (Coleoptera); and *Diprion pini* (Hymenoptera).

Thirty-three substances were tested. Most of them are anticoagulants on vertebrate blood *in vivo* or *in vitro*. These substances were arranged in the summarized report of the data below according to MacIntosh's (1949) classification of their mode of action on vertebrate blood. In addition, the disodium salt of ethylene diamine tetraacetic acid (Sequestrene NA 2: Alrose Chem. Co.: Dyckerhoff, Marx and Ludwig, 1942; Proescher, 1951), Apikur (Lenggenhager, 1949), hexamethylene-glycol (Foster, Samsa, Shulman and Ferry, 1951; Shulman and Ferry, 1951), the sodium salt of sulfated polygalacturonic acid methyl ester methyl glycoside or Treburon, Ro2–3053 (Mangieri, Engelberg and Randall, 1951), recently reported as anticoagulants of vertebrate blood, were also studied. We were unable to obtain hirudin and novirudin.

For study the reagents were dissolved or diluted at various concentrations with Meisenheimer's fluid, a special Ringer solution for insects (Bi-distilled water: 1000 gm.; NaCl: 7.5 gm.; $NaHCO_3$: 0.1 gm.; KCl: 0.2 gm.; $CaCl_2$: 0.2 gm.) This fluid was found to be a relatively good preservative of the cellular structures and did not interfere with the reactions. Distilled water was also used. The pH of the solutions was controlled before use.

Droplets of hemolymph issuing from the severed end of an antenna or a leg were allowed to fall in approximately the same volume of solution placed in the middle of a standard microscopical slide (Gold Seal glass). Preparations were also made by dipping the severed antenna directly into the solution. The mixture of hemolymph and solution, approximately 0.005 to 0.01 cc. in total volume, was homogenized by gentle lateral shakings of the slide. A thin Gold Seal coverglass, wet in its middle part with a droplet of the solution, was thus gently placed down onto the slide, droplet against drop of mixture. Spontaneous spreading gave a film of suitable thinness for observation. With this procedure, the labile hemocytes were generally protected from contact with glass surfaces before being thoroughly mixed with the solution of the inhibitor.

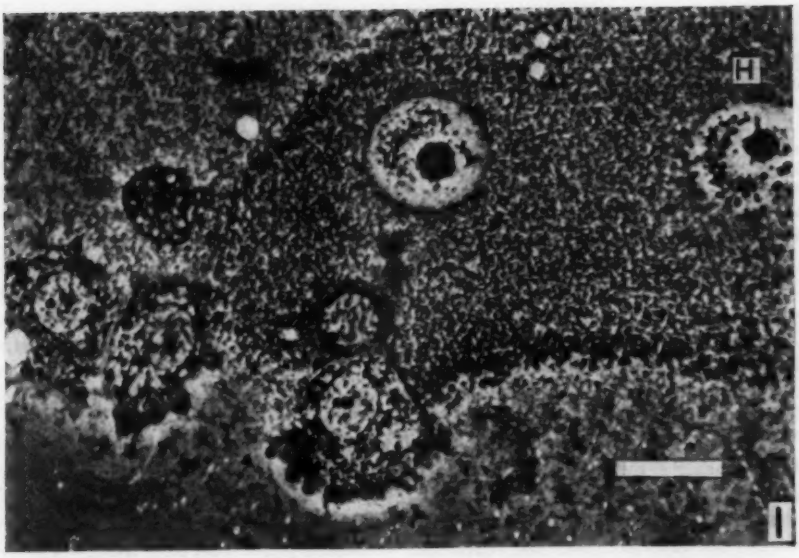
Control preparations were made with Meisenheimer's fluid alone at various pH values in order to appreciate the part played by the dilution of the hemolymph and by the pH in the modifications of the coagulation pictures.

Reactions of the hemolymph to contact with surfaces of various plastics (cellophane, acetophane, plexiglass, Lucite) or with glass made hydrophobic by coating with paraffin, formvar, parlodion and silicone G. E. Drifilm No. 9987, were also studied.

Silicone coatings of glass were prepared according to the standard technique used with vertebrate blood, Jaques *et al.* (1946). The preparations, stored in moist chambers, were observed during several hours.

Results

Nine hundred tests approximately were performed with the substances listed in the summarized report of the data (see below).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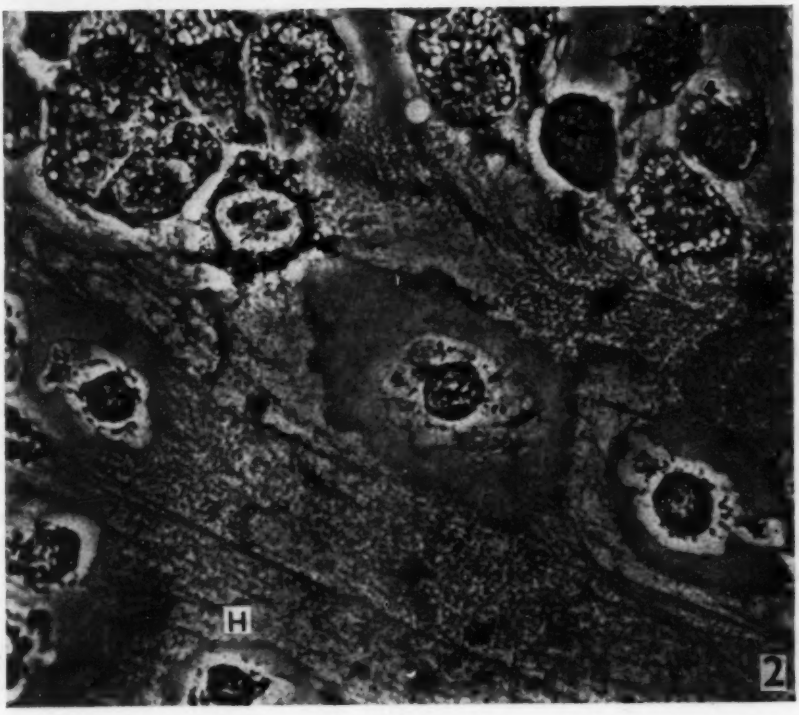
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Control of the effects of dilution

The degree of dilution of the hemolymph with the Meisenheimer's Ringer used in the present experiments (50%) did not interfere with the process of coagulation. The successive stages previously described in the normal hemolymph were also observed to take place after dilution. The highly labile hemocytes were characterized by a relatively small, sharply outlined nucleus, a pale hyaline cytoplasm, in which a few black granular particles were scattered. Within a few seconds, these hyaline hemocytes underwent a succession of intense modifications in their cytological structure: in the mole-cricket, for instance, the changes consisted of a rapid expansion of the cytoplasmic substance, with occasional beam-like bulging of cytoplasmic blisters and blebs (see Grégoire, 1951a; Figs. 2, 3, 6, 7, 8, 12). These alterations were accompanied or followed by the appearance in the surrounding plasma of a thin fog, developing into a circular cloud made of granular particles and progressively increasing in amount and in density. Variable extension of the process and organization of the coagulum into a delicate meshwork of fibrils took place subsequently (Figs. 1 and 2).

The difference between undiluted and diluted materials consisted chiefly of a decrease, proportional to the degree of the dilution, in the size of the islands and in the density of the coagulating materials (see Grégoire, 1951a; Figs. 1 and 2).

By spreading out between slide and coverslip, the diluted coagulum often assumed the shape of thin granular and elastic veil-like structures, a type of picture described elsewhere (Grégoire, 1951a; Figs. 24 and 26) in insect species belonging to various orders of insects (Coleoptera, Lepidoptera). The laying of the coverglass onto the slide often brought about a drawing of the coagulum into strings or threadlike fibrils. In the species used in the present studies, veils and strings were subsidiary morphological aspects induced by mechanical agencies.

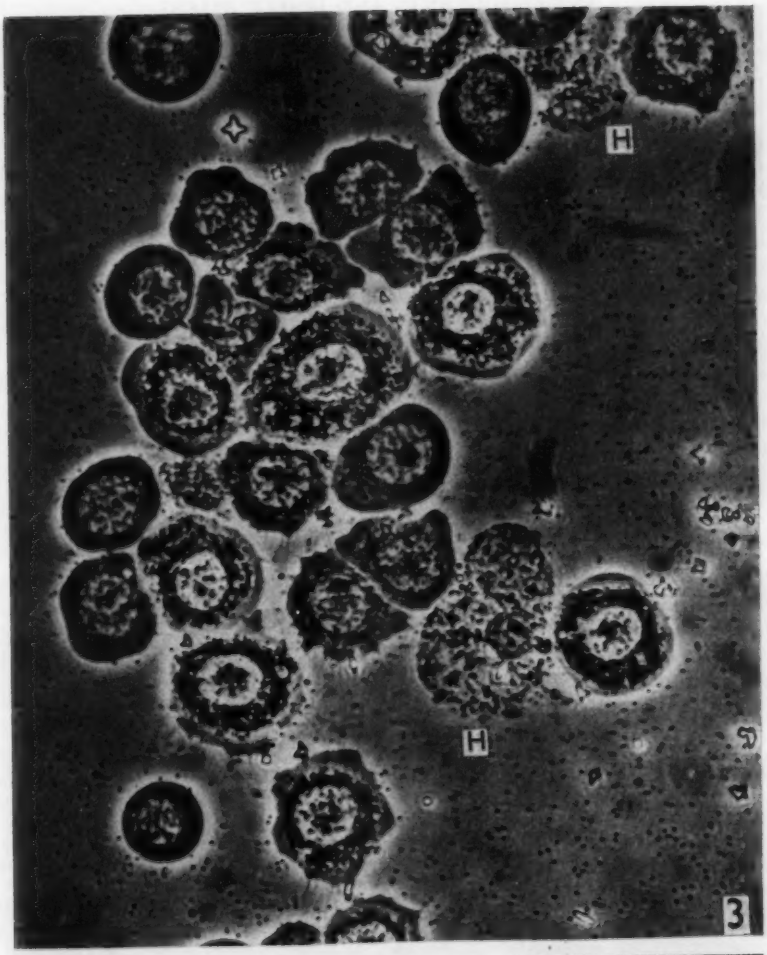
In most preparations, unless otherwise specified, one drop of hemolymph is added to one drop of a solution in Meisenheimer's Ringer of the substance to be tested, lying on a microscopical slide. The mixture, homogenized by lateral shaking of the slide, is allowed to spread out as a thin film between slide and coverglass.

All the microphotographs were taken by phase contrast microscopy (P. C. M. *Wild* M/10, Heerbrugg, Switzerland, with the objectives Ph 7/0.20 and Ph 40/0.66; Microcamera Leitz/Makam) and subsequently enlarged. The scale on the pictures represents 20 microns.

Variations in size of the hemocytes, as observed in the present pictures, are unreliable: they depend chiefly on adhesiveness factors varying with the amount of hemolymph, the area of spreading out, the thickness of the film and the local pressure of the coverglass onto the slide.

FIGURE 1. Mole-cricket (*Gryllota'pa gryllotalpa*, Orthopt.) Undiluted hemolymph, 20 minutes after withdrawal. Two hyaline hemocytes (H) are seen surrounded by a coagulation island. Extension of the process of coagulation at the upper left and lower right of the picture. Three granular hemocytes and two small round cells are being passively embedded in the extending granular coagulum. In the control preparations of the dilution factor, identical reactions can be observed, but some of the coagulation islands are of smaller size than in undiluted preparations (compare with Grégoire, 1951a, Figs. 1 and 2). × 800.

FIGURE 2. American cockroach (*Periplaneta americana* L., Orthopt.). Undiluted hemolymph, 12 minutes after withdrawal. Dense coagulation islands around several hyaline hemocytes. In the lower half of the picture, area of extension and of organization of the coagulum into a granular meshwork. About 13 elements, belonging to the other categories of hemocytes (granular, small round cells and transition forms) are passively embedded in the coagulum. × 800.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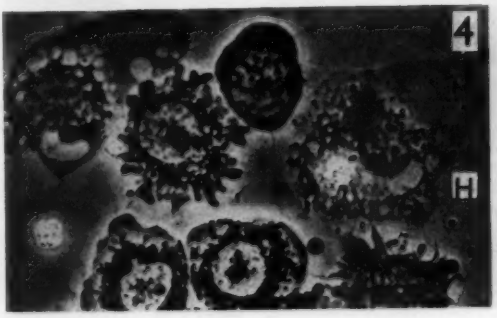
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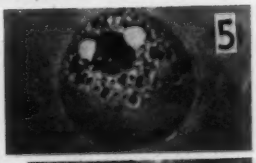
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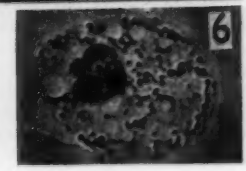
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Control of the factor pH of the solution investigated

Dilution of the hemolymph with Meisenheimer's fluid, brought to different pH covering the ranges of pH recorded in most solutions of the substances investigated, did not interfere with the process of coagulation. However, when hemolymph was diluted with Meisenheimer's fluid at pH 0.6 and 1.20, precipitates and a decrease in the amount of the coagulating materials were occasionally recorded. On the other hand, dilution with Meisenheimer's fluid at pH 9.45 appeared to induce an increase in the amount of the coagulum. This effect of alkalinity has already been pointed out (Muttkowski, 1924; Beard, 1950).

Microscopic aspects of the coagulation inhibition

The alterations taking place in the presence of potassium oxalate (at the concentration of 0.2% up) may be selected for the description of the total inhibition of the process of coagulation brought about by various compounds.

When the hemolymph was mixed with a solution of this substance, the hyaline hemocytes failed to undergo the alterations observed with the normal or the diluted blood. They appeared as pale spherical or oval cucumber-like bodies, loosely fixed to the glass or floating freely (Figs. 3 to 6, 15, 20 and 21).

Occasional rupture of these hyaline hemocytes, discharge of their cytoplasmic substance or granules did not bring about any change in their surrounding plasma; neither did contact of these elements (Figs. 18, 19) with foreign bodies, which in the normal blood rapidly induced the local formation of a coagulation island.

The other categories of hemocytes (small round or so-called stem cells, various kinds of granular hemocytes and transition forms), scattered or agglutinated in clusters at random, appeared also as spherical, oval or polygonal elements (Fig. 3). They occasionally spread out moderately and sent short spiky pseudopodia or bulging blebs (Fig. 4).

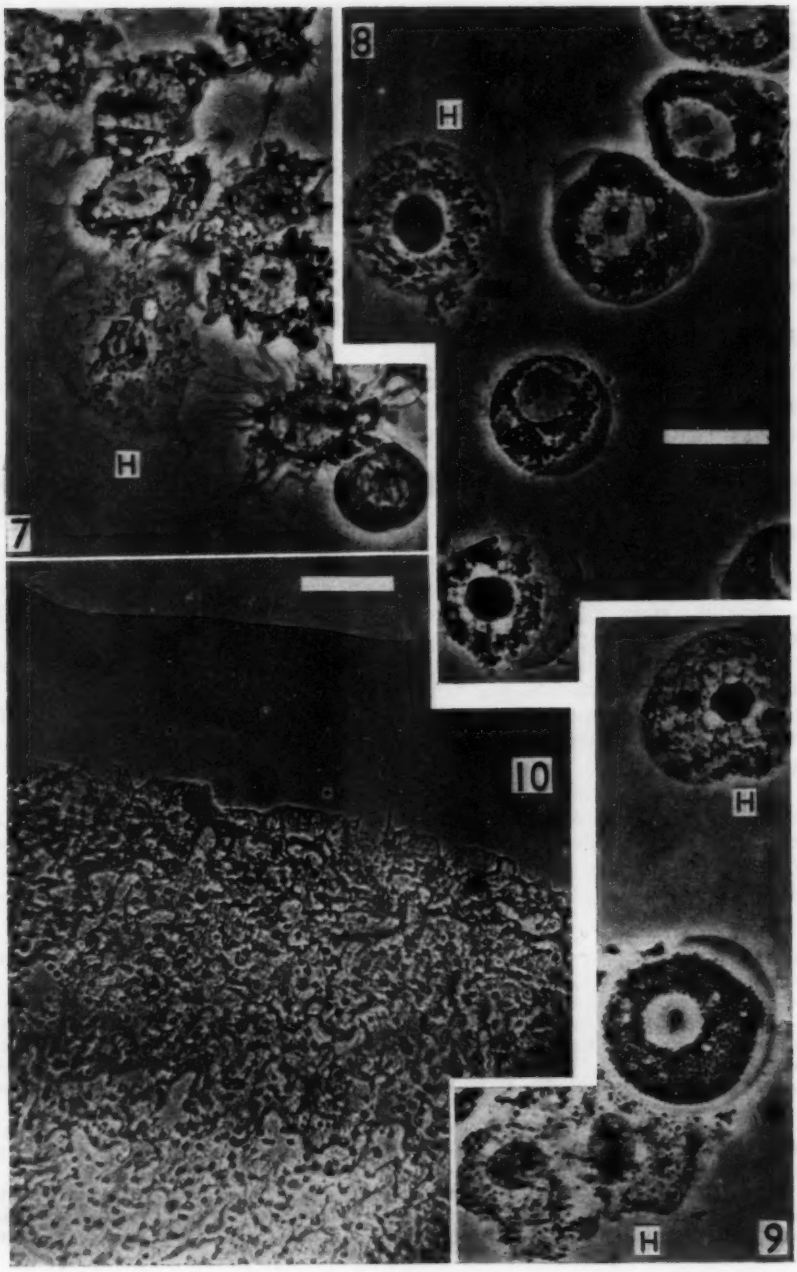
The oxalate type of reaction was observed in mixtures of equal amounts of hemolymph and solutions of potassium oxalate (0.2% up; Figs. 3 to 6, 11, 15, 20 and 21), sodium citrate (1%; Fig. 7), saturated magnesium sulfate, Sequestrene NA 2 (0.1% up; Figs. 12, 22, 23); of organic esters of sulfuric acid: Treburon [2] (1% up), Suramin (Belganyl: 0.1% up; Fig. 13); Bayer 205 (Germanine:

[2] A 0.1% solution of benzophenol, corresponding to the amount of antiseptic contained in the Treburon and heparin vials used, did not interfere with the coagulation process, in control preparations.

FIGURE 3. *Gryllotalpa*. Twenty-nine minutes after addition of three drops of hemolymph to one drop of a 1% solution of potassium oxalate (pH 7.60). No plasma reaction around two hyaline hemocytes. The other categories of cells are loosely agglutinated. Some send out thin spiky pseudopodia. The black dots in the plasma are precipitates of calcium oxalate. × 800.

FIGURE 4. *Gryllotalpa*. Hemolymph added to a 1% solution of potassium oxalate (pH 7.60) 80 minutes after mixture. No plasma reaction around a spread hyaline hemocyte. Two granular hemocytes with bulging blisters. × 800.

FIGURES 5 AND 6. *Gryllotalpa*. Hemolymph added to a 1% solution of potassium oxalate (pH 7.60) 28 and 60 minutes after mixture. No plasma reaction around two swollen hyaline hemocytes. Owing to the active motion of the intra-cytoplasmic granules, these structures lack sharpness in the picture. × 800.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0.2% up), Chicago Blue 6 B (0.1% up, with *Periplaneta* hemolymph; 1% up, with *Gryllotalpa* hemolymph; Fig. 14), Liquoid (1% up; Fig. 19), Chlorazol fast pink BKS (0.02% up); of organic bases and basic dyes: Methylene blue (2%), Janus green B (0.02% with *Periplaneta* hemolymph, 0.2% with *Gryllotalpa* hemolymph); of reducing substances: sodium bisulfite (2% up), sodium thiosulfate (2% up; Figs. 17 and 18), sodium hydrosulfite (3%). With 0.1% solutions of potassium oxalate and concentrations lower than 1% of sodium citrate (0.3%, 0.4%), definite inhibitory effects were recorded, but they were not constant.

Absence of coagulation was recorded with a 10% solution of Lanthanum chloride, with solutions of cocaine hydrochloride (1% up; Figs. 8 and 9) and with a 10% solution of DDT.

In mixtures of hemolymph with solutions of Heparin,[2] diethylamine, methyl violet, cysteine hydrochloride, glutathione, l-ascorbic acid, peptone, sodium taurocholate, Apikur, hexamethylene-glycol, a great deal of variation was recorded in the reactions, and anticoagulant effects of the oxalate type could not be consistently observed.

In several tests, there was a definite lack of coagulation, especially when using heparin or solutions at high concentration of acidified diethylamine (10%) alkalinized glutathione (10%), alkalinized cysteine hydrochloride (10%), peptone (10% up) and sodium taurocholate (16% up).

On the other hand, a 10% solution of cysteine hydrochloride (pH 0.60) seemed to increase the rapidity of initiation of the process of coagulation and the amount of coagulum.

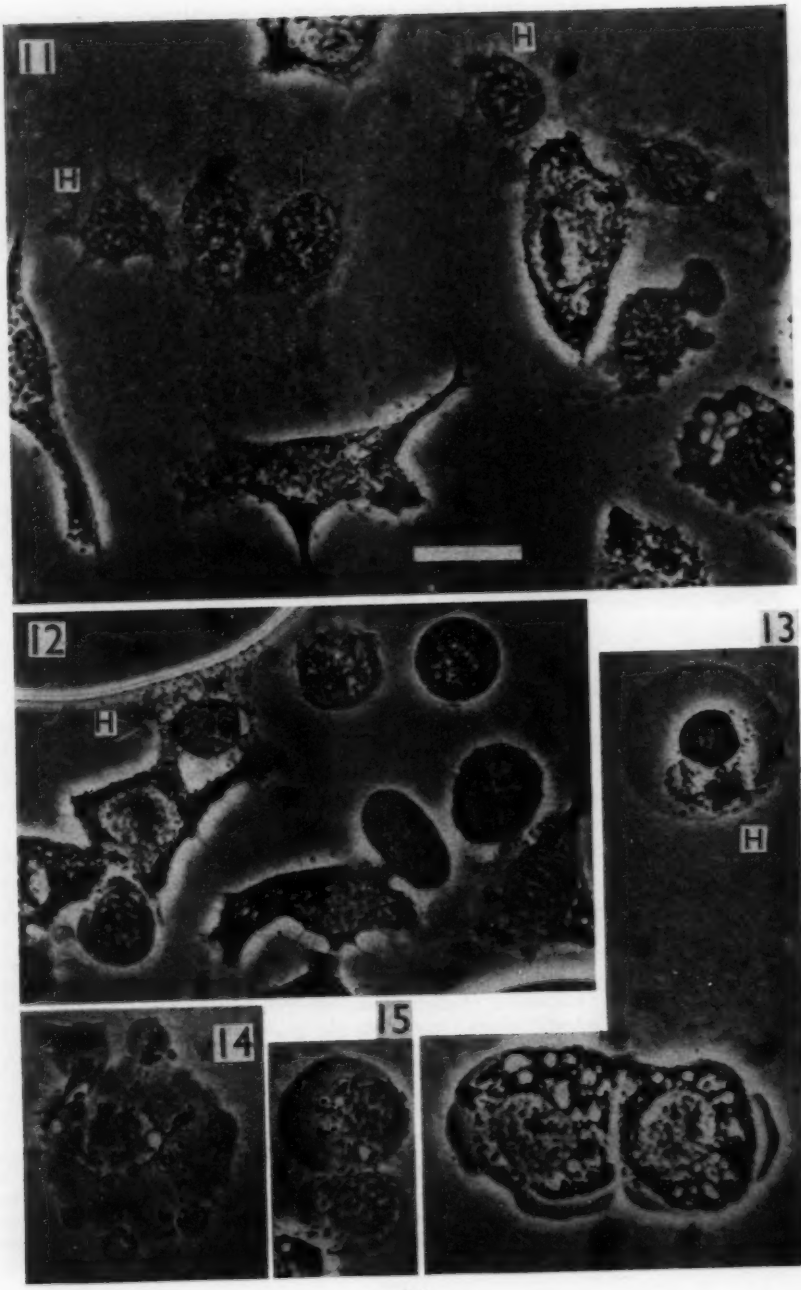
In other tests performed with the same substances, the process was not totally prevented, as in the oxalate type, but was definitely reduced; thin coatings of coagulating substance developed around a limited number of hyaline hemocytes; the clotted materials were decreased in amounts when compared with the corresponding formations in the dilution controls. Extension of the coagulation process from around the coagulation islands did not occur or was limited.

At the concentrations used in the present work, various solutions of saccharose, tricalcium phosphate, protamine, nucleic acid, sodium nucleinate did not interfere with the process of coagulation. However, pictures of inhibition were occasionally observed with solutions of sodium nucleinate (Fig. 16).

FIGURE 7. *Gryllotalpa.* Hemolymph added to a 1% solution of sodium citrate (pH 7.58) two hours after mixture. No plasma reaction around the hyaline hemocyte, containing intracytoplasmic granules in intense motion. Spreading out of the hyaloplasm of the granular hemocytes. × 800.

FIGURES 8 AND 9. *Gryllotalpa.* Ten and five minutes after addition of hemolymph to a 1% solution of cocaine hydrochloride (pH 6.67 and 6.60). All elements appear swollen and contain granules in active motion. No plasma reaction in spite of occasional (Fig. 8, H) discharge by the hyaline hemocyte of cytoplasmic substance and granules. × 800.

FIGURE 10. *Gryllotalpa.* Hemolymph clot between slide and coverglass coated with three sheets of Silicone G. E. Drifilm No. 9987, 105 minutes after withdrawal from antenna. A part of the contracting discoidal gel (see text) surrounded by a ring of fluid serum. At the borderline of the clot, the last retracting fibrils are seen. Within the clot, numerous little grey patches are coagulation islands centered by hyaline hemocytes. The scale represents 200 microns. × 70.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Special reactions of the hemolymph components to the different substances tested

Differences were observed in the degree of preservation of the cytological structures of the hemocytes, when the hemolymph was mixed with solutions of the substances investigated. A satisfactory preservation of the cell integrity was obtained with solutions of potassium oxalate, sodium citrate, Sequestrene and cocaine hydrochloride. Various degrees of damage to the cytological structures were occasionally recorded with heparin, Liquoid, Chicago Blue (2 to 15%). Solutions of sodium taurocholate (2–15%), hexamethylene-glycol (10–15%) and diethylamine (2% up) brought about dissolution of the formed elements. Milky precipitates were observed to develop frequently when the hemolymph was mixed with solutions of Liquoid, Janus green, methyl-violet and yeast nucleic acid.

In tests performed with diethylamine and protamine, the plasma reaction was characterized by the constitution of meshworks of thin elastic fibrils on which granular (precipitate) materials were absorbed.

Reaction of the hemolymph to hydrophobic surfaces

No modification nor delay in the process of coagulation was observed when the hemolymph was collected on non-adhesive surfaces of various resins and plastics (cellophane, acetophane, plexiglass, Lucite) or on glass coated with olive oil, paraffin, parlodion, formvar or with one and several sheets of silicone. Neither could coagulation be prevented when an oiled antenna was severed with oiled scissors or when the whole procedure of removing the blood was performed under oil.

When a coated coverslip was applied to the semi-spherical drop of hemolymph lying on a non-wetable slide, there was only a limited circular spreading out of the drop under the weight of the coverglass. Coagulation took place rapidly and the clot appeared as a thick elastic discoidal gel with sharp boundaries. This gel retracted progressively and within a few minutes it appeared surrounded by a ring of exuded serum (Fig. 10).

Discussion

Reliability of the procedure used

In the present study, the development or the absence of alterations in a category of hyaline hemocytes and in the plasma directly surrounding these elements was

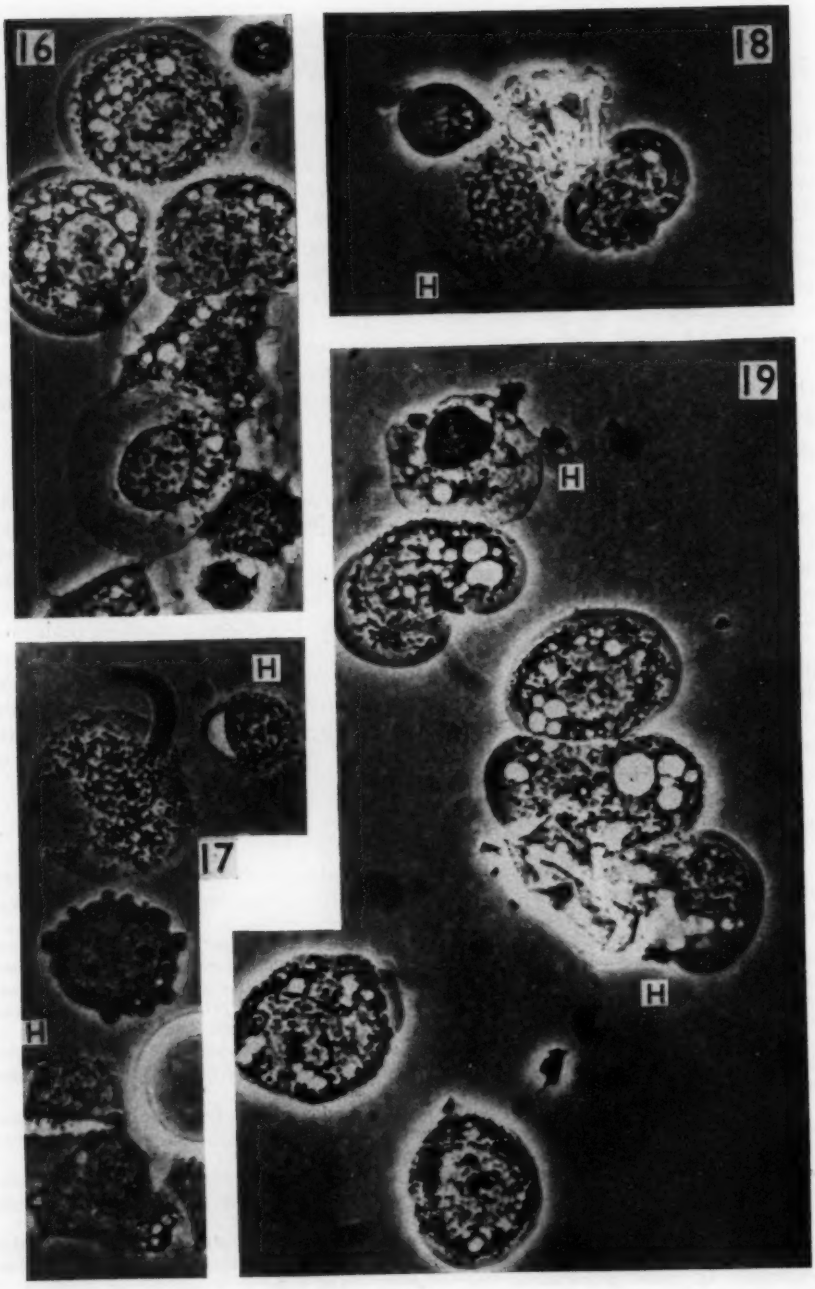
Figure 11. *Periplaneta.* Twenty-five minutes after addition of hemolymph to a 0.5% solution of potassium oxalate (pH 7.60). No plasma reaction around several hyaline hemocytes with unsharp cytoplasmic outlines or disintegrated to naked nuclei surrounded by fragments of cytoplasm. Elements belonging to other categories of hemocytes have undergone various modifications (lappet-like pseudopodia, vacuoles, cytoplasmic buddings). × 800.

Figure 12. *Periplaneta.* Twenty minutes after addition of hemolymph to a 0.1% solution of Sequestrene NA 2 (diluted from a 2% solution at pH 4.10). No plasma reaction around the hyaline hemocytes. × 800.

Figure 13. *Periplaneta.* Forty minutes after addition of hemolymph to a 0.1% solution of Belganyl-Suramin, diluted from a 2% solution at pH 7.37. A hyaline hemocyte and two granular hemocytes. No plasma reaction. × 800.

Figure 14. *Gryllotalpa.* Sixty-five minutes after addition of hemolymph to a 1% solution of Chicago Blue 6 B (pH 8.66). No plasma reaction around an altered hyaline hemocyte (peripheral cytoplasmic blisters). × 800.

Figure 15. Praying mantis (*Mantis religiosa,* Orthopt.). Forty minutes after addition of hemolymph to a 1% solution of potassium oxalate. No plasma reaction around two hyaline hemocytes. In control preparations (see Grégoire, 1951a, Figs. 9, 10 and 11) all elements of this category are surrounded rapidly by dense coagulation islands. × 800.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the test selected for appreciating the anticoagulant effects of different substances on insect hemolymph.

In spite of this rather simple criterion the following pitfalls could conceivably interfere with a correct appreciation of the results:

1) The extreme speed of coagulation of shed hemolymph which characterizes different insects constitutes a serious handicap in any experimental study of the process in these animals (Beard, 1950). This property of the insect blood is related to the high degree of lability exhibited by the hyaline hemocytes (coagulocytes) to contact with foreign environment (Grégoire and Florkin, 1950; Grégoire, 1951a): in these conditions, coagulation can easily take place at the wound site before hemolymph reaches the solution of inhibitor or is thoroughly mixed with it (Beard, 1950). In the latter case, microscopic areas of normal coagulation can develop at the site of falling of the drop into the anticoagulant solution, especially when hyaline hemocytes, still bathed in their normal plasma environment, accidentally meet tiny foreign bodies, such as dust particles or pieces of chitinous debris. Dipping antennas or legs into the solution before severance, immediate shaking to insure dispersion of the formed elements and thorough contact between the labile hemocytes and the inhibitor were found to decrease the occurrence of these accidents. This procedure, however, prevents accurate evaluation of the amount of blood flowing into the solution.

2) Stirring of the mixtures by shaking before the coverglass is applied does not completely prevent local variations in the degree of plasma dilution in different areas of the film spread out between slide and coverslip. Absence of islands of coagulation around hyaline hemocytes in the areas of higher dilution, usually in the peripheral parts of the preparations, can give deceptive pictures of inhibition. Attempts to appreciate this factor were performed in mock preparations, in which solutions of colloidal dyes (*e.g.*, india ink) were used instead of hemolymph and submitted to the same mechanical agencies. The distribution under the coverglass of the areas with various densities of the diluted dye was recorded, the stirring repeated on similar tests until films of uniform tone were obtained, and the same mechanical procedure applied to the diluted hemolymph.

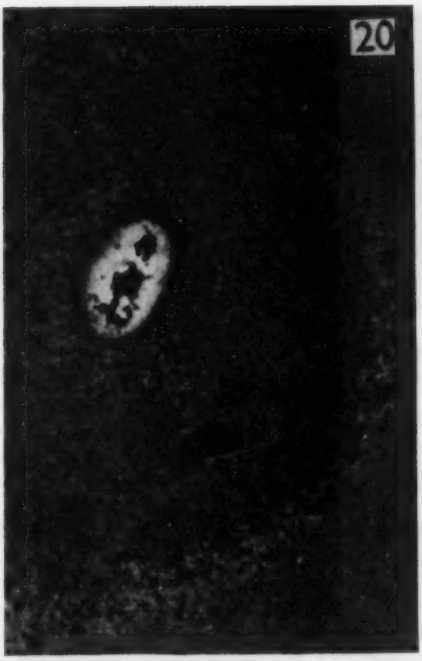
3) The present results have been established on standard preparations consisting mostly of approximately equal amounts of hemolymph and solutions to be tested. It is possible that decreasing the relative amount of hemolymph or increasing that of the solutions would allow one to observe anticoagulant effects for substances for which none has been recorded in the present conditions.

FIGURE 16. *Periplaneta.* One hundred and ten minutes after addition of hemolymph to a 2% solution of sodium nucleinate Merck (pH 5.50). Area without plasma coagulation (exceptional). Seven hemocytes including a swollen hyaline hemocyte. × 800.

FIGURE 17. *Periplaneta.* Sixty minutes after addition of hemolymph to a 2% solution of sodium thiosulfate in distilled water (pH 6.25). No plasma reaction around hyaline hemocytes. The large hyaline cell with moving intra-cytoplasmic granules belongs probably to the same category. × 800.

FIGURE 18. *Periplaneta.* Preparation as in Figure 17, after 70 minutes. There is no plasma reaction around the hyaline hemocyte anchored to a foreign body (piece of chitinous debris). In normal hemolymph, contact with a foreign body would rapidly induce production of a coagulation island around such a hyaline hemocyte. × 800.

FIGURE 19. *Periplaneta.* Twenty-five minutes after addition of hemolymph to a 1% solution of Liquoid (pH 7.60). No plasma reaction around two hyaline hemocytes, one of them attached to a foreign body. × 800.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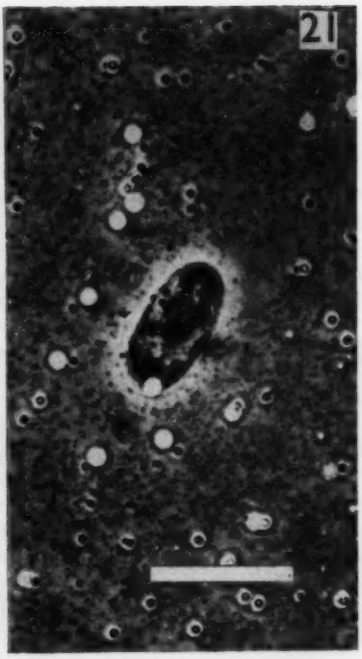
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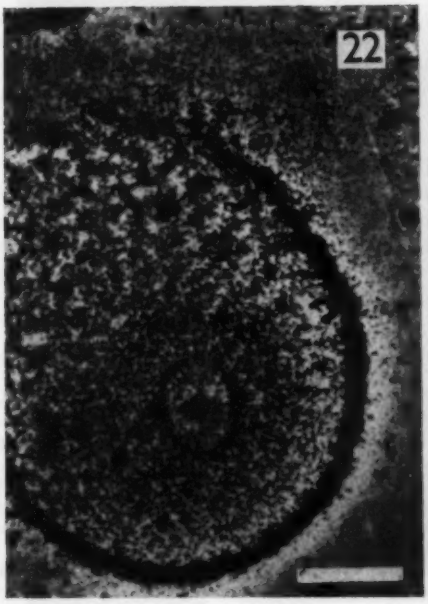
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In addition, the threshold of efficiency of various substances seems to vary with the species of insect used: for instance, as seen in different tests, for preventing coagulation in *Gryllotalpa* hemolymph, it was often necessary to use higher concentrations of anticoagulant solutions than for obtaining inhibition of the coagulation of *Periplaneta* hemolymph, with the same substance.

Comparative study of the literature

Coagulation of insect hemolymph has been reported to be prevented by various organic acids: nucleic (Paillot, 1923), citric and ascorbic acids (Beard, 1950), vapors of acetic and several fatty acids (Shull, Riley and Richardson, 1932; Fischer, 1935; Shull, 1936); also by surface-active and physical agents, such as heating of the animal body (Fredericq, 1881; Yeager, Shull and Farrar, 1932; Babers, 1938; Beard, 1950), freezing and ultrasonic waves (Beard, 1950).

On the other hand, failure of several anticoagulants of mammalian blood to inhibit the coagulation of the insect hemolymph has been reported for magnesium sulfate, (Fredericq, 1881), potassium oxalate (Muttkowski, 1924; Yeager, Shull and Farrar, 1932), heparin, hirudine, citrates, dicoumarol (*in vivo:* Beard, 1950). Hemolymph coagulation was not affected by cyanide fumes (Muttkowski, 1924; Shull, Riley and Richardson, 1932), nor by calcium cyanide (Beard, 1950).

The present results are at variance with the former data obtained with potassium oxalate, citrate and to some extent with heparin.

The discrepancies could be explained by differences in the experimental conditions (for instance, concentrations and relative amounts of the inhibitors used). As the literature on the process of normal coagulation in insects is controversial, it is more probable that the standards used for determining an anticoagulant effect cannot be compared.

The normal hemolymph coagulation of different insects has been described as being either a cellular agglutination or a plasma coagulation, both considered as two physiologically distinct processes, which can occur independently or together in the same insect or in different insects (see Beard, 1950).

In a recent paper, Beard (1950) refers to the difficulties encountered in appreciating the effects of the coagulation inhibitors on insect blood, while with the clotting of mammalian blood, fibrin formation is a convenient end-point for judging

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FIGURE 20. Water scorpion (*Nepa cinerea,* Hemipt.). Undiluted hemolymph, 25 minutes after withdrawal. A large circular island of coagulation is seen surrounding an altered hyaline hemocyte. × 1080.

FIGURE 21. *Nepa.* Forty minutes after addition of hemolymph to a 1% solution of potassium oxalate (pH 8.10). A cucumber-like inert hyaline hemocyte is seen floating freely in the fluid plasma. Numerous grey and black dots are moving precipitates of calcium oxalate. × 1080.

FIGURE 22. *Lethocerus cordofanus* Mayr (Belostomatidae from the Belgian Congo). Undiluted hemolymph, 25 minutes after withdrawal. A hyaline hemocyte is seen in the center of a circular island of coagulation. × 800.

FIGURE 23. *Lethocerus.* Eighty minutes after addition of hemolymph to a 0.5% solution of Sequestrene NA 2. No plasma reaction. A group of variously altered elements belonging to different categories of hemocytes. Owing to the shrinkage, hyaline hemocytes are difficult to recognize unquestionably: two pale cells (lower part of the picture) and two elements isolated in squares are probably hyaline hemocytes. × 800.

the effect. According to Beard, dispersion of the formed elements is the desired response in searching for an inhibitory agent.

As it appears from our previous and present studies, a process of cellular agglutination does not play any part in the phenomenon of hemolymph coagulation in the species of insects used in the present experiments. The categories of blood cells other than the hyaline hemocytes are inert, scattered or agglutinated at random and passively embedded in the plasma coagulum. Accordingly, the dispersion of the formed elements could not be selected as a test for measuring an inhibiting effect on the process of hemolymph coagulation in insects.

The present results, on the other hand, are in agreement with various data obtained with crustacean blood, as regards the inhibiting effect on the true plasma coagulation (so called second coagulation) of magnesium sulfate (Halliburton, 1885; Ducceschi, 1903, 1915), potassium oxalate (Bottazzi, 1902; Ducceschi, 1903, 1915; Loeb, 1903, 1904; Nolf, 1909; Parsons and Parsons, 1923), highly concentrated solutions of peptone (Bottazzi, 1902; Loeb, 1903; Nolf, 1909; Parsons and Parsons, 1923), sodium hydrosulfite (Hensill, 1948) and cocaine hydrochloride (Ducceschi, 1903, 1915).

Mechanism of the coagulation inhibiting effect in insect hemolymph

Little is known of the chemical nature of the substances involved in the process of hemolymph coagulation in insects. The terms fibrin and gelatine used in the literature for characterizing different aspects of the insect clots rather represent morphological analogies with the corresponding compounds in crustacean or vertebrate blood.

An anticoagulant can act on insect hemolymph either on the hyaline hemocytes involved in the initiation of the process or on the coagulable compounds contained in the plasma, or on both factors.

From the present data, different anticoagulants can be efficient in preserving the extremely labile hyaline hemocytes from the alterations which they rapidly undergo in normal hemolymph when they come in contact with glass surfaces and foreign particles and which distinguish this category of blood elements from the other less labile hemocytes. In mixtures of hemolymph and efficient inhibitors, the hyaline hemocytes generally keep a spherical or oval shape and do not spread out. Inconstant anticoagulants are unable to prevent completely the alterations of all the hyaline hemocytes and the subsequent reaction of the plasma in their vicinity: the coagulation process is scattered and the areas of extension of the coagulum are decreased by various degrees.

As often suggested for a corresponding category of elements in crustacean blood (Halliburton, 1885; Hardy, 1892; Ducceschi, 1903; Tait and Gunn, 1918) the hyaline hemocytes of insects could take part in the coagulation process by yielding coagulation—inducing substances. Anticoagulants would prevent the release of these active substances. However, in incidental observations, no clotting reaction could be detected in the plasma around some hyaline hemocytes which had discharged a part of their cytoplasmic substance or were reduced to naked nuclei after their rupture. This result could be explained as well by inactivation of the coagulation-inducing substances contained in these hemocytes as by alterations of one or more plasma components involved in the coagulation process.

Of six compounds which act on vertebrate blood as removers of calcium ions, deionizing agents or preventers of platelet disintegration, five were efficient anticoagulants of insect hemolymph. The present results do not allow one to conclude whether these compounds interfere with the insect coagulation process either like other strong salts, in preventing the release of active substances by the hyaline hemocytes, or in inactivating these substances, or by removing calcium salts possibly involved in the mechanism of the clotting process.

In preliminary experiments, a calcium chloride solution was allowed to flow into a film of oxalated hemolymph. When the calcium chloride reached the spherical, inert hyaline hemocytes bathed in the oxalate solution, these elements underwent sudden modifications, consisting of shrinkage or the extension of adhesive pseudopodia. A definite reaction was not observed in the plasma directly surrounding these elements. Owing to the formation of precipitates between potassium oxalate and calcium chloride, interfering with the observations, the development of veil-like elastic structures in different areas of the preparations, suggesting a possible clotting reaction, remains questionable and requires further study.

Among the organic esters of sulfuric acid, which were found to be efficient anticoagulants of insect blood in the present study, it is worth mentioning that the trypanocidal drug suramin would be an inhibitor of enzymatic activity, according to a recent finding (Willis and Wormall, 1950).

Several other anticoagulants of vertebrate blood, listed above, were not inhibitors at the concentrations used or brought about various degrees of interference with the process of coagulation.

Owing to the lack of data on the number and on the chemical nature of the coagulation factors in insects, interpretation of the failure of these anticoagulants of vertebrate blood to inhibit the process in insects cannot be given at the present time.

Comparative physiology

The present results offer additional data with regard to the existence of converging functional characters in zoological groups as distant as arthropods and vertebrates: vertebrate platelets (Ferguson, 1934), Hardy's explosive corpuscles in crustacea (Hardy, 1892; Ducceschi, 1903, 1915; Tait and Gunn, 1918) and hyaline hemocytes in insects (Grégoire and Florkin, 1950; Grégoire, 1951a) exhibit specific morphological changes, which are related to the initiation of the plasma coagulation. Substances decreasing, delaying or preventing the alterations in the platelets (Bizzozero, 1882; Bürker, 1904; Wright, 1941; Feissly and Ludin, 1949), in the crustacean explosive corpuscles (Ducceschi, 1903, 1915; Tait and Gunn, 1918), and in the hyaline hemocytes of insects, as shown in the present studies, decrease, delay or prevent the blood coagulation. However, there are differences between vertebrate platelets and insect hyaline hemocytes; the latter elements do not exhibit, at least *in vitro*, the property of auto-agglutinability characterizing the platelets, and their fragility seems to be much greater. In the present investigations, the hyaline hemocytes were not preserved on hydrophobic surfaces, even when they were protected by oily environment from contamination with the tissues at the wound, before falling onto the silicone-coated surface. The oil procedure was successful in the hands of Tait and Gunn (1918) in preventing on plain glass

the alterations in the explosive corpuscles and the plasma coagulation in *Astacus* blood.

Consistent failure of the silicone-coated surfaces to prevent coagulation of insect hemolymph seems to indicate that different factors are probably responsible for the induction of the alterations in insect hyaline hemocytes and vertebrate platelets.

Substances tested and reactions of insect hemolymph. Summarized report of the data

For brevity's sake, the reference marks used here below record the following reactions of the plasma:

+: development of coagulation islands around hyaline hemocytes (coagulocytes), followed by various degrees of extension of the process of coagulation;

−: no coagulation, all hyaline hemocytes inert, loosely fixed to the glass or floating in the fluid plasma;

*: development of coagulation islands or veils in limited areas, obviously accidental, induced in the vicinity of foreign bodies, or at the site of falling down of the drop of hemolymph into the solution of anticoagulant (see text);

$: heterogeneous reaction. Areas without coagulation (oxalate type), areas with various degrees in the reduction of the process, as compared with the control preparation in Meisenheimer's fluid. Plasma reaction consisting of tiny coagulation islands or fringes around the hyaline hemocytes, and of thin veils without or with poor extension.

The concentrations of the solutions used are given in %. Unless otherwise indicated, the pH values are those of solutions in Meisenheimer's fluid. The preparations consisted of approximately equal volumes of solution investigated and of hemolymph. The total volume of mixture in each test amounted approximately 0.005 to 0.01 cc. The mixtures were spread out into films under 24 × 24 mm. and 24 × 30 mm. coverslips (see methods above).

1. *Controls of the factors dilution and pH of the solutions of the substances investigated.*

Meisenheimer's fluid (incidentally distilled water).

pH 0.6, 1.20, 3.30, 4.12, 4.32, 5.63, 5.83, 7.28, 7.38, 7.83, 8.00, 9.45: 160 tests, all +.

2. *Salts, removers of calcium ions and deionizing agents.*

Potassium oxalate.

0.07%: 3 tests $ and 1 test − *.
0.1%: 2 tests $ and 2 tests − *.
0.2%: 8 tests −.
0.5%, 1% (pH 7.60–8.10), 4%, 10% (pH 7.80–8.00) and 20%: 32 tests −.

Sodium citrate.

0.1%: 3 tests $ and 2 tests −.
0.3%: 3 tests $ and 3 tests − (incidentally *).
0.4%: 2 tests $ and 6 tests − (incidentally *).
1% (pH 7.58): 14 tests − (incidentally *).

Magnesium sulfate.
Saturation (pH 5.86) : 5 tests −.
Sequestrene NA 2.
0.1%, 0.5%, 1% and 2% (pH 4.10) : 23 tests − (incidentally *).
Saccharose (in Meisenheimer's fluid).
2%, 5%, 10% (pH 7.21) and 50% (pH 7.27) : 7 tests + and 5 tests $.
Saccharose (in distilled water).
1% (pH 6.57), 10% (pH 6.40) and 50% (pH 6.20) : 8 tests + and 12 tests $.
Tricalcium phosphate.
1% (pH 6.51) : 2 tests +.

3. *Organic esters of sulfuric acid.*

Heparin (*Liquemine* Roche).
50 anticoagulant units per drop used (pH 6.50) : 3 tests +, 17 tests $ and 10 tests −.
Heparin (powder dissolved in Meisenheimer's fluid).
4800 Toronto anticoagulant units per cc. : 5 tests $ and 3 tests −.
2400 Toronto anticoagulant units per cc. : 2 tests +.
Treburon Ro 2–3053/B–3.
1% (*Periplaneta*) : 6 tests − (incidentally *).
2% (*Periplaneta*) : 6 tests − (incidentally *).
1.5% (*Gryllotalpa*) : 1 test $ or − and 1 test −.
3.75%, 7.5% and 15% (*Gryllotalpa* and *Blabera*) : 16 tests − (incidentally *).
Suramin (Belganyl).
0.01% : 2 tests $.
0.1%, 0.2% (in distilled water : pH 6.26), 2 % (in Meisenheimer's fluid : pH 7.37) and 10% (in distilled water : pH 6.20) : 12 tests −.
Bayer 205 (*Germanine*).
0.02% : 2 tests + (*Gryllotalpa*) and 4 tests $ (*Periplaneta*).
0.2% and 2% (pH 7.50) : 12 tests −.
Chicago Blue 6 B.
0.1% : 4 tests + (*Gryllotalpa*) and 4 tests − (*Periplaneta*).
1% (pH 8.66) and 10% (pH 8.61) : 18 tests −.
Liquoid.
0.01%, 0.02% (pH 7.65), 0.1% (in distilled water : pH 6.43 and in Meisenheimer's fluid : pH 7.82–7.96) : 11 tests +.
0.5% : 2 tests $ and 2 tests −.
1% (pH 7.60) : 6 tests −.
2% (in distilled water : pH 6.40) : precipitates, 3 tests −.
Chlorazol fast pink BKS.
0.005% : 2 tests +.
0.01%, 0.02%, 0.05%, 0.2%, 0.5% and 2% (pH 6.00) : 7 tests $ and 21 tests − (precipitates).

4. *Organic bases and basic dyes.*

Protamine.
0.1% and 1% (in distilled water: pH 1.83) : 8 tests +.
Diethylamine.
0.1% (in distilled water: pH 9.29) : 6 tests + and 2 tests −.
0.5% (in distilled water: pH 10.61) : 6 tests $.
1% (in distilled water: pH 11.07 and in Meisenheimer's fluid: pH 11.83) : 2 tests $, 8 tests −. In 2 other tests, dissolution of the hemocytes.
1% (acidified to pH 6.00) : 2 tests +, 4 tests $ and 2 tests −.
10% (pH 12.27) : 3 tests $ and dissolution of the hemocytes.
10% (acidified to pH 5.80) : 8 tests −.
Methylene Blue.
0.02% and 0.1% (pH 7.38) : 2 tests +, 8 tests $ and 2 tests − *.
1% (pH 3.30) : 4 tests $ and 2 tests −.
2% (pH 3.30) : 6 tests −.
Janus Green B.
0.02% : 2 tests $ (*Gryllotalpa*) and 6 tests − (*Periplaneta*).
0.2% (in distilled water: pH 3.89 and in Meisenheimer's fluid: pH 5.38) and 2% (pH 2.66) : 9 tests − and precipitates.
Methyl violet.
0.1% and 0.3% : 2 tests +, 1 test $ and 5 tests −.
1% (pH 6.62) : 2 tests: precipitates.

5. *Reducing substances.*

Sodium bisulfite.
2% (pH 4.49) and 5% (pH 4.30) : 8 tests −.
Sodium thiosulfate.
2% (pH 6.25) and 10% (pH 5.92) : 8 tests − (in 2 tests *).
Sodium hydrosulfite.
3% (pH 5.50) : 2 tests −.
Cysteine hydrochloride.
0.2% and 1% : 3 tests +.
2% (pH 1.10) : 5 tests + and 6 tests $.
2% (alkalinized to pH 8.30) : 8 tests − (in 4 tests *).
10% (pH 0.60) : 5 tests +, 2 tests $ and 2 tests −.
10% (alkalinized to pH 6.92) : 8 tests−.
Glutathione.
0.2% and 1% : 3 tests + and 1 test $.
2% (pH 2.70–2.85) : 3 tests + (*Periplaneta*), 10 tests $ (*Periplaneta*) and 8 tests − (*Gryllotalpa*).
2% (alkalinized to pH 7.30) : 8 tests + (*Gryllotalpa*), 8 tests $ (*Periplaneta, Gryllotalpa*) and 8 tests − (*Periplaneta*).
10% (pH 2.61) : 2 tests + and 5 tests $.
10% (alkalinized to pH 8.79) : 4 tests −.
l-ascorbic acid cryst.
2% (pH 2.90) : 2 tests $ and 8 tests − *.
10% (pH 2.20) : 5 tests $ and 3 tests −.

6. *Salts of rare earth metals.*

Lanthanum chloride.

0.2% and 1%: 10 tests + (precipitates).
5%: 2 tests $ and 2 tests − *.
10%: 6 tests − (incidentally *).

7. *Miscellaneous substances.*

Peptone (Merck 7213).

2% (in distilled water: pH 7.34 and in Meisenheimer's fluid: pH 7.66): 10 tests $.
5%: 2 tests −.
10% (in distilled water: pH 7.58 and in Meisenheimer's fluid: pH 7.38): 4 tests $ and 9 tests −.
16% (pH 7.60): 5 tests −.

Sodium taurocholate.

0.1%, 0.5%, 2% (pH 7.00–7.17), 5% (pH 7.02), and 10%: 3 tests + and 20 tests $.
16% (pH 6.99): 10 tests − (dissolution of the hemocytes).

Cocain hydrochloride.

1% (pH 6.17 − 6.67) and 5%: 18 tests − (incidentally *).

Sodium nucleinate (Merck).

2% (pH 5.48) and 10% (pH 4.99): 6 tests + and 4 tests $.

Yeast nucleic acid (Merck).

0.025%, 0.5%, 1%, 2% (pH 3.00) and 10% (pH 3.48): 13 tests + and in 1 test, precipitates.

Apikur (bee venom).

0.015%, 0.03% and 0.07%: 6 tests +, 9 tests $ and 1 test −.
0.15% (pH 3.40): 8 tests $ and 1 test −.

Hexamethylene glycol.

2% (in distilled water: pH 5.60 and in Meisenheimer's fluid: pH 6.96), 5% (in distilled water: pH 5.50 and in Meisenheimer's fluid: pH 6.96): 20 tests + and 2 tests $.
10% (pH 6.78): 3 tests $ and 9 tests − (incidentally *).
15% (pH 7.27): dissolution of the hemocytes in all tests.

D. D. T.

10%: 8 tests − (incidentally *).

Summary

1. The effects of 33 substances, most of them anticoagulants of vertebrate blood, have been investigated *in vitro* on the hemolymph of different species of insects, chiefly Orthoptera, exhibiting in normal conditions a conspicuous process of coagulation.

2. The tests (900 approximately) consisted of mixtures in equal amounts of hemolymph and solutions at various concentrations in a special Ringer for insects (Meisenheimer's fluid) of the substances studied. The mixtures were observed by means of a phase contrast microscope after they were spread out in thin films between slide and coverglass.

3. The part played in the reactions by the dilution of the hemolymph and by the pH of the solutions used was appreciated in control preparations made with Meisenheimer's fluid alone at various pH values; no interference with the coagulation process was recorded within the range of pH used (0.60 to 9.45).

4. The morphological criterion selected for appreciating an anticoagulant effect was the development or the absence of alterations in the category of hemocytes especially involved in the initiation of the plasma coagulation, and the development or the absence of coagulation islands around these cells.

5. From the 33 substances tested, 18 were efficient anticoagulants in relatively small amounts; among them were four salts, removers of calcium ions or de-ionizing agents of vertebrate blood, six organic esters of sulfuric acid, including trypanocidal drugs, two basic dyes and three reducing substances. Consistent anticoagulant effects could not be obtained with heparin, and with solutions of the following substances (unless at high concentrations and, for some of them, after acidification or alkalinization): diethylamine, cysteine hydrochloride, glutathione, l-ascorbic acid, peptone, Apikur and hexamethylene glycol.

6. Different hydrophobic surfaces, including glass coated with Silicone G. E. Drifilm No. 9987, did not induce any definite modification in the coagulation process.

7. A tentative explanation of the anticoagulant effects is given in the discussion; when the substances tested prevent the morphological alterations in the category of hyaline hemocytes involved in the process of coagulation, this process does not occur. The results bring additional support to the previous interpretation that the hyaline hemocytes play an important part in the initiation of the hemolymph coagulation in different insects.

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STUDIES ON SHELL FORMATION. II. A MANTLE-SHELL PREPARATION FOR IN VITRO STUDIES[1]

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This report describes a preparation, consisting of an oyster mantle and its associated shell, which has been developed for studying mechanisms of shell formation *in vitro*. The preparation has been found capable of depositing shell of normal structure and can be easily maintained for periods of several days. An account of shell formation by the mantle-shell preparation is presented.

Methods

All experiments were carried out with the oyster *Crassostrea virginica* Gmelin.[3] The mantle-shell preparation is isolated in the following manner. The oyster is placed on its left valve and the hinge is gently pried open with an oyster knife or scalpel. The knife is then twisted in the hinge in order to open the valves slightly, and the adductor muscle is severed in the middle with a razor blade. The opening of the valves should be kept at a minimum during this operation so that the mantle is disturbed as little as possible. Unlike many of the bivalves, the mantles of the oyster are fused along the base of the gills. The right valve is lifted slightly, and beginning at the posterior end and working toward the hinge, the right mantle is snipped free from the left mantle, the gills, and the visceral mass with small scissors. The organs attached to the left mantle can next be removed. One now has the two valves with their attached mantles completely intact. When the dissection is performed with sufficient care both mantles remain attached to the shell and retract only slightly. On being placed in sea water the mantles relax and cover the entire shell surface (Fig. 1).

The mantle-shell preparations were suspended in running sea water by means of a plastic V-shaped clamp with the mantle downward. In this position mucus is eliminated more efficiently.

To establish the ability of the mantle to deposit shell *in vitro*, two or three fragments of glass coverslips were inserted between the mantle and the shell following the method of Brooks (1905) for the whole oyster. This can be conveniently performed by lifting a small portion of the mantle edge with a small spatula and inserting a coverslip fragment. Even in the inverted position, the coverslip fragments were firmly pressed against the shell surface by the mantle. The coverslip fragments were removed after the desired interval, thoroughly washed in distilled water to remove dissolved salts, and examined under the light field and polarizing microscopes.

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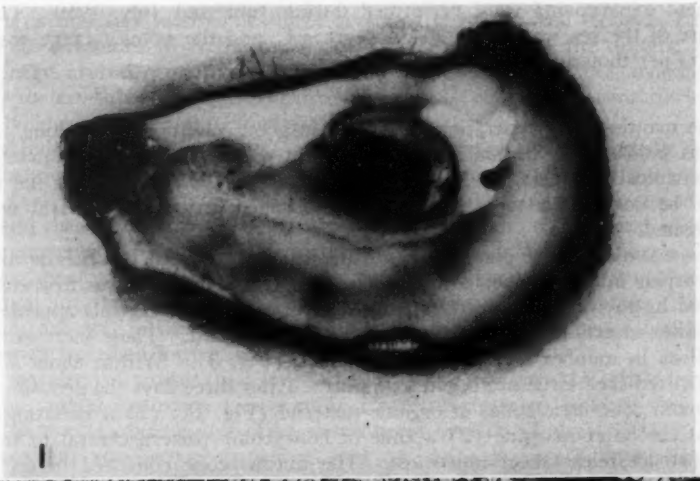
[3] Previously *Ostrea virginica*. Re-named by Gunter (1950).



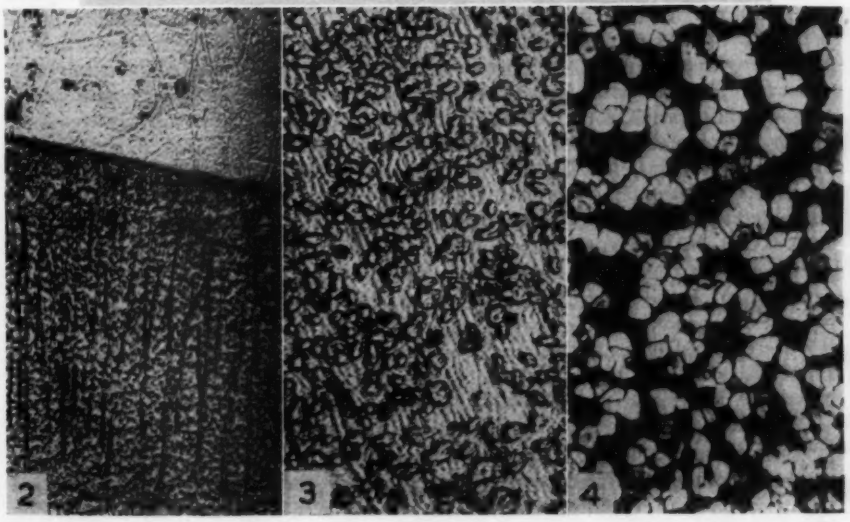


FIGURE 1. Mantle-shell preparation of a right shell.

FIGURE 2. Area below the diagonal line represents deposits by the mantle-shell preparation on a coverslip fragment over a period of 62 hours. The coverslip was inserted between the mantle and the shell 74 hours after its isolation. The marginal fibrous zone is clearly distinguishable, but the other two zones cannot be easily distinguished without the aid of the polarizing microscope.

FIGURE 3. Crystalline deposits of the innermost zone (structureless). Lower zone of Figure 2 magnified.

FIGURE 4. The zone of honeycomb pattern photographed between crossed Nicol prisms. Intermediate zone of Figure 2 magnified.

These experiments were performed during June and July, 1950. The temperature of the sea water was 26–30 degrees C. and the salinity range was 31 to 36 parts per thousand.

Results

The mantle-shell preparations remained in good condition for as long as seven days, as indicated by normal ciliary movement and retraction of the mantle edge on mechanical stimulation. More recently, Jodrey (1953) has found the respiration to be normal for the same duration. Longer periods of survival were not investigated.

The examination of pieces of coverslip inserted between the mantle and shell after various intervals revealed that a sheet of organic material was first elaborated followed by the deposition of birefringent crystals. These crystals appeared eight hours after insertion and produced bubbles in dilute acid. These increased in size as well as in number and became contiguous (Fig. 3). Within about 2½ days an organized shell structure began to appear. After three days the deposits showed three main concentric zones of organic material (Fig. 2): (1) a radiating fibrous zone at the outer margin; (2) a zone of honeycomb pattern central to this; and (3) a structureless sheet innermost. The fibrous zone consisted of an organic sheet with radiating fibers running parallel to the surface and perpendicular to the periphery of the mantle. The honeycomb zone showed cellular-like structure probably with open upper surfaces since some of the small areas contained crystals whereas others did not. Except for the absence of fibers the innermost zone resembled the fibrous zone. The fibrous and honeycomb structures were much thicker than the structureless organic sheet and together measured about one mm. in width where oysters of 10 cm. in length were used. The thickness of the organic deposits gradually decreased toward the center of the shell. The three zones resembled the structure of the periostracum, prismatic and nacreous layers of normal shell except that the polyhedral units of the periostracum were lacking.

Birefringent crystalline materials were deposited on all three zones, the most dense being on the honeycomb area. As a result, this region is the most distinct of the three regions when observed between crossed Nicol prisms (Fig. 4). Crystals were imbedded in the cellular framework of the honeycomb region, but were simply scattered on the other two zones. With treatment in 0.001 N HCl the crystalline material dissolved while the organic structures remained intact.

Not all of the preparations were capable of depositing crystals within the 8-hour period mentioned, but pieces of coverslip left in place for more than 40 hours were rarely without crystalline deposits. By introducing the glass fragments at intervals it was found that crystal deposition was possible even after four days of isolation. However, the time required for deposition was longer than with a recently isolated mantle. While birefringent crystals were deposited by all areas of the mantle, the rate of deposition near the muscle was slower than near the margin. This was confirmed later by the use of Ca^{45} (Jodrey, 1953).

Discussion

Studies of shell formation *in vivo* by Bevelander and Benzer (1948), confirming the observations of earlier workers, showed that the organic matrix is first de-

posited as a thin sheet and comes to be arranged in prisms and striae in later development. Granules from the mucus cells are deposited in the organic matrix and undergo crystal growth and continue to grow eventually into arrangements whereby the crystals come to be enclosed in a thin layer of organic matrix and assume a polyhedral shape (in *Pinna*).

Bevelander and Martin (1949) reported that isolated strips of mantle of *Pintata radiata* can be maintained *in vitro* and will produce crystals in the course of several days. In the mantle-shell preparation described here the association of mantle and shell of the intact oyster is maintained; and both organic and crystalline shell components are elaborated in patterns closely resembling the normal. This synthetic capacity, together with the stability *in vitro*, emphasizes the utility of the preparation for the study of shell-forming mechanisms of the mantle. The potentialities are still further increased by the use of radioisotopes for quantitative measurements of shell formation (Wilbur and Jodrey, 1952).

The mantle-shell preparation forms shell more slowly than the whole oyster as shown by crystal formation on coverslip fragments inserted beneath the mantle and by the rate of calcium deposition as measured by Ca^{45} (Jodrey, 1953). Other differences may also become evident as shell formation *in vitro* is studied in more detail. Differences between the mantle-shell preparation and the intact oyster must be considered a fortunate circumstance, for by their study and correction through experimental procedures, information concerning factors operative in the formation of shell should result.

The author is indebted to Dr. K. M. Wilbur of Duke University for his advice on the experimental work and critical review of this paper. He also wishes to express his thanks to Dr. W. A. Chipman and Dr. C. E. Atkinson of the U. S. Fish and Wildlife Service, Beaufort, N. C., for providing laboratory facilities and experimental material.

Summary

1. A preparation consisting of an oyster mantle and its attached shell has been devised for *in vitro* studies of shell formation. Shell consisting of typical organic matrix and birefringent crystalline material was elaborated by this preparation, though at a slower rate than in the intact oyster.

2. All parts of the mantle were shown to be capable of shell formation *in vitro*.

3. The isolated mantle-shell preparation can be maintained in good condition for several days.

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STUDIES ON SHELL FORMATION. III. MEASUREMENT OF CALCIUM DEPOSITION IN SHELL AND CALCIUM TURNOVER IN MANTLE TISSUE USING THE MANTLE-SHELL PREPARATION AND Ca^{45} [1, 2]

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As a prerequisite to a study of the mechanisms of shell formation, the suitability of the isotope method for measuring shell growth in the intact oyster has been studied and reported in the first paper of this series (Wilbur and Jodrey, 1952). With the use of Ca^{45}, shell growth taking place in a few hours could be detected and measured. The isotope method also made possible a study of the distribution of newly deposited calcium on the inner shell surface.

With the radioisotope method for measuring rate of calcium deposition in the whole oyster established, it was desirable as a next step to eliminate some of the variables of the whole oyster and to direct more attention to the mantle itself. Bevelander and Martin (1949) have shown that excised strips of mantle will deposit calcium; and Hirata (1953) has found that a preparation consisting of the intact mantle of the oyster with its attached shell will survive for several days and will deposit both the organic matrix and the calcite of shell. This latter preparation is valuable in that it eliminates the complexity of the whole animal and yet maintains the integrity of the mantle-shell association. The mantle-shell preparation has been studied here with respect to its capacity to deposit calcium, and the rate has been compared with that of the whole oyster.

The mollusc mantle has long been considered as the tissue immediately responsible for the secretion of shell; and it was indeed surprising to find that the Ca^{45} of mantle tissue of whole oysters and mantle-shell preparations in labeled sea water was extremely low, even though a relatively large amount of the isotope was being deposited in the shell. This condition of low activity of the mantle and high activity of the shell was suggestive of two mechanisms. The calcium deposited in the shell might not actually enter the mantle at all; or, a very small amount of calcium might enter the mantle and turn over (be renewed) at a very rapid rate and then become deposited in the shell. To test these hypotheses the turnover rate of calcium in the mantle was studied in the mantle-shell preparation which, in limiting the source and fate of calcium, provides a simpler system than the whole oyster.

[1] Aided by a grant from the Atomic Energy Commission.

[2] The author is deeply indebted to Mr. C. E. Atkinson and Dr. W. A. Chipman of the U. S. Fish and Wildlife Service at Beaufort, N. C., who provided laboratory facilities which made this study possible.

METHODS

Rate of calcium deposition in the mantle-shell preparation

Oysters of the species *Crassostrea virginica* were collected near Beaufort, N. C., during the latter part of June, 1951, and maintained near the laboratory in natural waters. The species, habitat and methods of collection and maintenance of these oysters were the same as those used in immediately preceding experiments on the whole oyster (Wilbur and Jodrey, 1952).

The mantle-shell preparations were prepared as described by Hirata (1953). Each preparation was then placed in one liter of sea water in a large finger bowl and aerated. Some of the mantles retracted during dissection; and after a time when they had again spread out over the shell, Ca^{45} was added to the water. The added isotope was carrier-free in the form of the chloride. The activity was 3.45 microcuries as standardized with a calibrated Co^{60} reference source. The containers were covered with water-proof paper to prevent loss during aeration. The temperature of the water, though nearly constant for each experiment, varied from 25° C. to 28.2° C. during the entire experimental period. The salinity of the water used ranged from 34.84 to 35.61 parts per thousand.

After intervals of 8, 12 and 24 hours, the preparations were removed and the radioactive calcium deposited on the shells was measured as previously described (Wilbur and Jodrey, 1952). In experiments of 24 hours duration the radioactive sea water was changed at the end of 12 hours. Measurements of radioactivity of circular areas of 6.2 $cm.^2$ were made on the anterior, center and posterior regions of the inner shell surface.

Calcium turnover rate in the mantle

For the study of calcium turnover rate in the mantle each mantle-shell preparation was placed in 500 ml. of aerated sea water with a Ca^{45} activity of 5.8 microcuries until the Ca^{45} in the mantle reached equilibrium, *i.e*, the specific activity became maximal. After this period (usually four hours) in radioactive sea water, the preparations were placed in an equal volume of sea water containing no Ca^{45} and allowed to remain there for intervals from 10 to 130 minutes so that the rate of disappearance of Ca^{45} from the mantle could be determined. On removal from sea water the mantle edge [3] was dissected from the remainder of the mantle; both portions were rinsed briefly in sea water, drained for 10 seconds on filter paper, after which each was weighed with a torsion balance and placed in a separate glass counting cup. The tissue was then digested in the same cups with fuming nitric acid and dried with a heat lamp for subsequent measurement of Ca^{45}. Ca^{45} deposited on shells was also determined as previously outlined (Wilbur and Jodrey, 1952).

A more complete examination of the uptake of Ca^{45} by the mantle was also carried out by placing mantle-shell preparations in radioactive sea water, removing at intervals of 10 minutes to 8 hours and carrying out the procedure as above.

[3] The term "mantle edge" refers to the thickened, tentacled, pigmented border of the mantle. Because this portion differs macroscopically, histologically and histochemically from the remainder of the mantle, it is not unreasonable to suppose that the two portions have different roles in shell formation which might be reflected in different calcium turnover rates. Hence, the two portions of the mantle were treated separately.

Twelve mantle-shell preparations were used in each experiment. In six experiments the temperature varied from 26.1° C. to 27.9° C., while the salinity range was 35.16 to 35.90 parts per thousand.

Total calcium content of mantle tissue was determined for mantle edge and mantle interior on pooled samples from 16 oysters. The tissue was dry-ashed (at a temperature not high enough to cause loss of Ca) to a white residue which was then dissolved in dilute HCl, diluted to 200 ml. and the calcium determined by a method which excludes magnesium and the alkali metals (Kolthoff and Sandell, 1943).

Results

Calcium deposition in shell

The shells of the mantle-shell preparations became rapidly radioactive in radioactive sea water, measurable amounts of Ca^{45} being deposited in one hour (not included in graphs). As in the whole oyster, the highest rate of deposition is in the posterior region of the shell. After 24 hours exposure to 3.45 microcuries of Ca^{45}, the mean deposition of Ca^{45} in some 20 shells expressed in counts per minute per 6.2 cm.^{2} of shell was 657 for posterior, 407 for center and 182 for anterior regions. Figure 1 shows the rate of calcium deposition in the posterior region of shell of 37 mantle-shell preparations. As in the whole oyster, a high degree of variability is found in rate of calcium deposition in the mantle-shell preparation also, but the mean rate is very nearly linear.

An approximation of the total amount of calcium deposited after a given period of time on the inner shell surface can be made by comparing the average activity of the whole surface of the shell with the activity of the sea water medium which contains a known amount of calcium. (See Wilbur and Jodrey, 1952, for details and limitations of the method.) An approximation of the average activity of the entire surface can be made by averaging the activity of the three regions measured. The calculated amount of calcium deposited during 24 hours by a mantle-shell preparation of an oyster about 8 cm. long is 1.27 mg. Calculated by the same method, the amount of calcium deposited by the whole oyster was 11.08 mg. for the same period.

Calcium turnover rate in the mantle

Data for the rate of uptake of Ca^{45} by the mantle edge are plotted in Figure 2, in which Ca^{45} per gram of tissue is given in counts per minute as a function of hours of exposure to radioactive sea water. It is apparent that the Ca^{45} content rises very rapidly. The greater part of the uptake is completed after about one-half hour, while the maximum amount of Ca^{45} has been taken up after two to four hours. A comparison of the specific activity of mantle calcium and sea water calcium allows one to calculate the amount of calcium which has been renewed in the mantle during this interval. The method of calculation follows. At the maximum (and equilibrium) the average Ca^{45} activity is about 7×10^2 counts per minute per gm. wet weight. The calcium content of this weight of mantle edge is 0.95 mg. as determined by analysis for total calcium. Correcting the mantle edge activity to the same geometry used for measurement of sea water activity, the specific activity of the mantle edge calcium is 7×10^{-4} microcuries per mg. The specific

activity of the sea water calcium is 2.9×10^{-2} microcuries per mg. The specific activity of the mantle edge calcium at equilibrium is 2.4 per cent of the specific activity of the sea water which means that 2.4 per cent of the mantle calcium has been renewed. Since it is not possible to further increase the specific activity of the calcium in the mantle, it must be concluded that the greater portion of the mantle

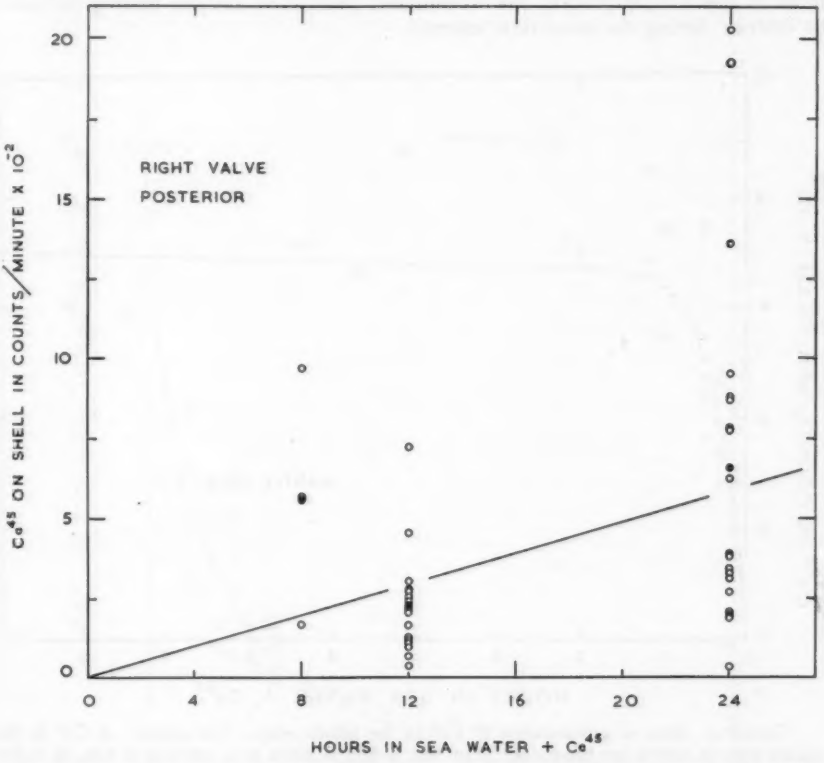

FIGURE 1. Rate of calcium deposition in the mantle-shell preparation. Ca^{45} deposited on a 6.2 $cm.^2$ area in the posterior region of the shell is plotted as a function of hours of exposure to radioactive sea water. (Original counts have been multiplied by 10^{-2} in order to use a one-digit scale.) Each point represents a measurement on a different individual. The mean rate is very nearly linear.

calcium is inert and only a small percentage, 2.4 per cent, is being renewed. Since the mantle edge contains 0.95 mg. calcium per gm. wet weight, the portion being renewed is 2.4 per cent of 0.95 mg. or 0.023 mg.

Ca^{45} uptake by the mantle interior follows much the same pattern as shown in Figure 2, but the Ca^{45} content at equilibrium is about one-half as great as that in the mantle edge. Since there is only about one-half as much total calcium in this

part of the mantle, the specific activity is therefore the same as in the mantle edge. The figure of 2.4 per cent for the actively participating calcium accordingly holds for mantle interior also.

Disappearance of Ca^{45} from the mantle edge is extremely rapid (Fig. 3), the greater fraction being removed during the first half hour, indicating a very high rate for renewal of calcium. The disappearance of calcium from the mantle interior is also a rapid process, the accumulated radioactive calcium leaving the mantle interior during the same time interval.

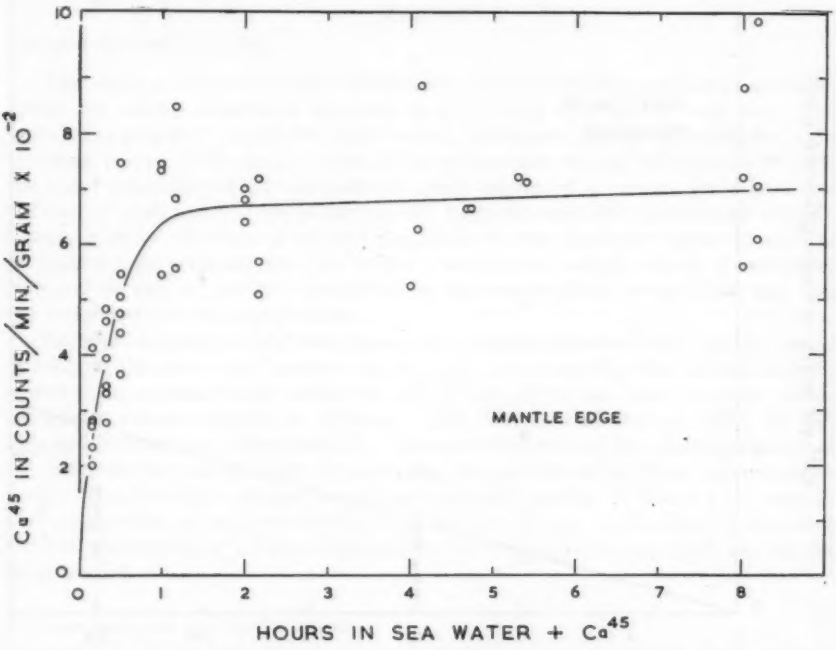

FIGURE 2. Rate of accumulation of Ca^{45} in the mantle edge. The amount of Ca^{45} in the mantle edge in counts per minute per gram wet weight is given as a function of time in radioactive sea water. Each point represents a measurement of Ca^{45} in an individual mantle. Most of the uptake is completed after one-half hour. After two to four hours the mantle has taken up as much Ca^{45} as it will accumulate.

The rates of disappearance of Ca^{45} from mantle edge and mantle interior are exponential functions. From a semi-log plot of the data (Fig. 4) the half-time (period of time required for one-half the Ca^{45} to leave the mantle) is found to be 16.5 minutes. The turnover time (see Hevesy, 1948 and Siri, 1949) is 16.5/0.693 or 24 minutes. The turnover rate, which is the actual weight of calcium disappearing and being replaced per unit time, is 0.023/24 or 1×10^{-3} mg. per minute. In 24 hours the calcium turnover in one gram of mantle edge would be 1.44 mg. A similar calculation gives a calcium turnover of 0.62 mg. per gram of mantle

interior. The complete mantle of one oyster of the size used weighs about 1.5 grams, the mantle edge comprising about 0.5 grams of the total. Hence the calcium renewed per day in the whole mantle would be $1.44/2 + 0.62$ or 1.34 mg. The estimated amount of calcium deposited on shell in the same period as measured from

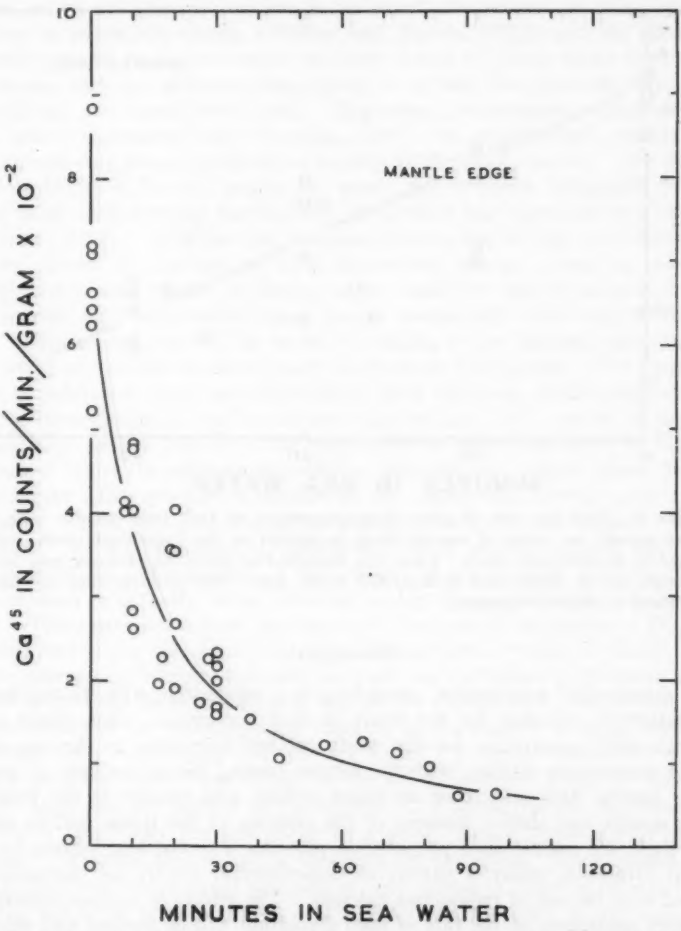

FIGURE 3. Rate of disappearance of Ca^{45} from the mantle edge when placed in non-radioactive sea water. After the mantles had taken up as much Ca^{45} as they would accumulate, they were placed in non-radioactive sea water and the rate of loss of the isotope from the mantle was determined by measuring the Ca^{45} content of the mantle after increasing intervals. Each point is for a separate mantle. The rate of disappearance is very rapid; most of the Ca^{45} has disappeared after one-half hour. Although individual variability exists, the curve is an exponential function with time.

counts of radioactivity of shell was 1.27 mg. This figure was calculated from the average activity of the inner shell surface, the rate of calcium deposition being lower in the central region of the shell which is in keeping with the lower calcium turnover rate of the mantle interior.

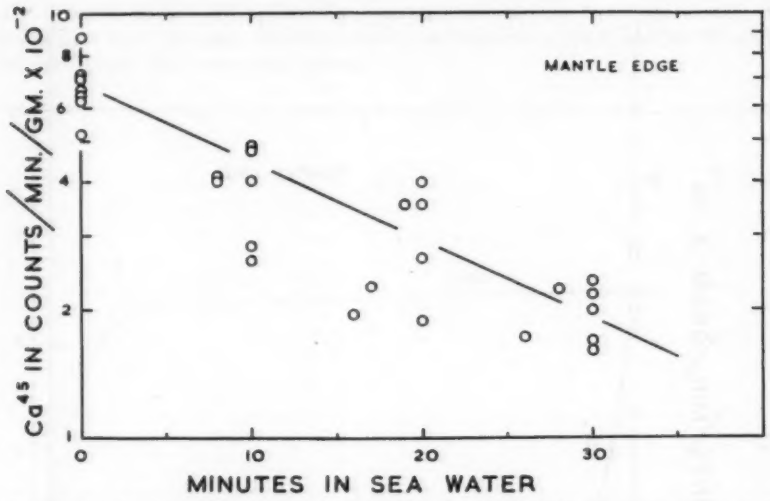

FIGURE 4. Semi-log plot of rate of disappearance of Ca^{45} from mantle edge. Ca^{45} in counts per minute per gram of mantle tissue is plotted on the logarithmic scale and time is represented by an arithmetic scale. From this straight line curve the turnover time for calcium in the mantle can be determined as described in the text. The turnover time for the calcium being renewed is about 24 minutes.

DISCUSSION

The mantle-shell preparation, consisting of a right valve with its attached mantle, is extremely valuable for the study of shell formation. One might compare the mantle-shell preparation for the study of shell formation to the use of tissue slices for metabolism studies, with the former having the advantages of remaining alive for several days and being an intact system with respect to the relationship between mantle and shell. Because of the viability of the tissue and its ability to deposit shell, the mantle-shell preparation provides a convenient system for studying shell formation under a variety of experimental conditions, especially when combined with the use of radioactive calcium. The effects of various inhibitors and respiratory substrates on the rate of shell formation will be studied and reported in another paper in this series. Since this preparation limits the source and fate of mantle calcium, its use, combined with the isotope technique, allows one to study directly the path taken by calcium which becomes deposited as shell.

This present study has shown that the mantle of the mantle-shell preparation will deposit as shell substance calcium from the surrounding sea water. Experimental evidence that the calcium of shell enters the mantle directly from the sea

water has not been at hand, although suggestions that it may do so have been made (Galtsoff, 1934; Robertson, 1941). Apparently, at least part of the calcium deposited as shell does not require the participation of digestive and circulatory systems; although Bevelander (1952), reporting on uptake of Ca^{45} by intact molluscs, states that calcium ions present in the water are ingested by the organism and are localized in several organs. Although an exact comparison of rates of calcium deposition in the whole oyster (Wilbur and Jodrey, 1952) and the mantle-shell preparation cannot be made because the latter work was done about a month later in the season, and at a different temperature, it is clear that the isolated mantle deposits calcium at a much lower rate. Neglecting discrepancies which may enter due to season (Loosanoff and Nomejko, 1949), the mantle-shell preparation deposits calcium only about one-ninth as rapidly as the whole oyster. The pattern of deposition, however, is very nearly the same; and it seems reasonable to assume that the usual shell-forming mechanisms are present but operating at a lower rate (see Hirata, 1953). Whether the decreased rate is due to lack of a more efficient transport system for calcium or to a diminished energy supply is not known. Possibly an adequate source of energy might make the rate of calcium deposition in the mantle-shell preparation more nearly comparable with that of the intact animal. This assumption will be tested by adding to the medium respiratory substrates which are known to affect mantle respiration (Hilgartner, 1951; Jodrey and Wilbur, unpublished data) and determining their effect on calcium deposition.

In the introduction it was mentioned that the low Ca^{45} content of the mantle tissue and high isotope content of the shell could be made compatible by one of two mechanisms: either the calcium deposited as shell did not actually enter the mantle cells or a very small amount of calcium in the mantle was turning over at a very rapid rate. The results obtained in this study of turnover rate support the second supposition. By a comparison of specific activity of mantle calcium with the specific activity of sea water calcium, it was shown that only about 2.4 per cent of the mantle calcium is actually being renewed under the experimental conditions described. The remainder is inert and does not move out of the mantle. The amount of calcium renewed in the mantle during a 24-hour period would be about 1.34 mg., while the amount of calcium deposited on shell was calculated to be about 1.27 mg. It would seem from these two figures that in the mantle-shell preparation at least, the greater part of the calcium being renewed in the mantle is being deposited as shell, rather than exchanging with the sea water. It seems reasonable to assume that this situation would also obtain in the whole oyster. Thus, in spite of the relatively high calcium content of the mantle (2.5 times that in sea water), shell deposition is apparently brought about by a small portion turning over at a very rapid rate. Whether this would be true also for the whole oyster cannot be said, although the consistently small amounts of Ca^{45} found in mantles of intact oysters depositing relatively large amounts of the isotope in the shell would indeed point to this.

The large amount of calcium in the mantle which appears to be inert is of considerable interest. Molluscan mantles have been observed by several workers to contain granules of calcium phosphate (Hayasi, 1939; Biedermann, 1914; Bevelander and Benzer, 1948) or conglomerates of calcium and organic material (Hayasi, 1938). These large granules may perhaps constitute the inert portion.

Under certain conditions of the whole oyster, such as long periods of closure when the acidity of body fluids increases (Dugal, 1939), this calcium may be mobilized. This could be tested experimentally by an examination of the mantle for granules after periods of forced closure. Combined autoradiographic and histochemical studies of mantle sections might also aid in a more accurate localization of inert and dynamic calcium.

The author wishes to thank Dr. Karl M. Wilbur, whose suggestions and criticisms have been most helpful during both the experimental work and preparation of the manuscript.

Summary

1. A study of calcium turnover and calcium deposition by the oyster mantle in the absence of other soft tissues has been carried out using mantle-shell preparations of *Crassostrea virginica*.

2. The mantle of the mantle-shell preparation is able to deposit calcium from the sea water in shell. At least part of the calcium deposited as shell substance does not require the participation of digestive and circulatory systems.

3. The rate of deposition in the mantle-shell preparation was about one-ninth that of the whole oyster under the conditions of the experiment, but the distribution of newly deposited calcium was similar in both.

4. The greater portion of the calcium in the mantle appears to be inert, since the specific activity of the mantle calcium could not be increased beyond a small percentage of the specific activity of the sea water calcium. A small fraction (2.4 per cent) was renewed every 24 minutes, the turnover rate being 0.6 micrograms of calcium per minute per gm. of mantle.

5. The calcium turnover rate in the mantle edge is approximately twice as rapid as in the mantle interior but since the latter is renewing a calcium reservoir which is only about one-half as great as the former, the turnover time for the renewing fraction is the same in each.

6. Since the amount of calcium being renewed in the mantle is very nearly the same as the amount being deposited on the shell, it would seem that in the mantle-shell preparation at least, the fraction of rapid turnover brings about the formation of shell.

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THE FIBRILLAR SYSTEMS OF CILIATES AS REVEALED BY THE ELECTRON MICROSCOPE. I. PARAMECIUM [1]

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For many years the fibrillar systems and associated organelles of protozoa have attracted the attention of cytologists and protozoologists. Initially this interest appears to have been directed by morphological and functional considerations. More recently and with the appreciation of the self-duplicating nature of these systems, their causal relation to morphogenesis, regeneration and the polarity of the organism has commanded first attention (Tartar, 1941; Fauré-Fremiet, 1948; Lwoff, 1950; Weisz, 1951). Unfortunately, these studies have been limited by the fact that the structures in question have dimensions which border on the resolving power of the light microscope. Thus, with the exception of cilia, flagella and exploded trichocysts (Jakus and Hall, 1946; Pitelka, 1949; Finley, 1951, and others) the detailed anatomy of the fibrillar apparatus and associated organelles has remained obscure. For an adequate understanding of the functional and morphogenetic properties of these systems a detailed picture of their fine anatomy would appear to be essential. Presumably such a picture could be obtained by an electron microscope examination of these systems in serial sections (Sedar, 1952) or preferably after appropriate dissection of the material. In the present study "sonic dissection" was found to be a simple and effective method of preparing fragments of the ciliate protozoan surface, together with the subjacent fibrillar apparatus, for electron microscope examination. The present effort, the first in a series of morphological studies employing this technique, should now provide a new point of departure for the investigation of the physiology of locomotion, coordination and developmental mechanics in ciliate protozoa.

The primary fibrillar apparatus of ciliates consists of a number of parallel structural units, the kineties (terminology of Lwoff, 1950). Each of the several kineties in turn is composed of: 1) a longitudinal row of cilia; 2) a longitudinal row of small granules, one granule at the base of each cilium (the ciliary basal bodies or kinetosomes); and 3) a longitudinal ectoplasmic fiber which connects all the basal granules in the row (the silver line or neuromotor fiber, neuroneme, or kinetodesma). As revealed by the light microscope, this unit of organization is an anatomically continuous structure. In the more complex ciliates this fibrillar pattern is rendered more involved by modifications in the arrangement of the kineties, especially in the oral region, and by superposition of accessory organelles and fibers upon this system of kineties. In the most extensively studied ciliate, Paramecium, this complexity is evident. At least two other ectoplasmic fibrillar systems besides the primary one have been described and the detailed relationships

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of these are disputed (see Taylor, 1941, for review of fibrillar systems in Paramecium and other ciliates). In the oral region the arrangement of fibers and cilia becomes highly complex (Lund, 1933).

Most of the structures described by others using classical methods have been observed in the present study and their relationships have been clarified. However, attention has been directed mainly to the structure and organization of the primary fibrillar apparatus or kineties, with their component cilia, kinetosomes and kinetodesmas. The last are never found to be single "fibers" in the classical sense. As reported briefly elsewhere (Pitelka and Metz, 1952) a kinetodesma is seen to be a bundle of tapering fibrils, each arising independently from a kinetosome and extending for a modest distance along the bundle.

Materials and Methods

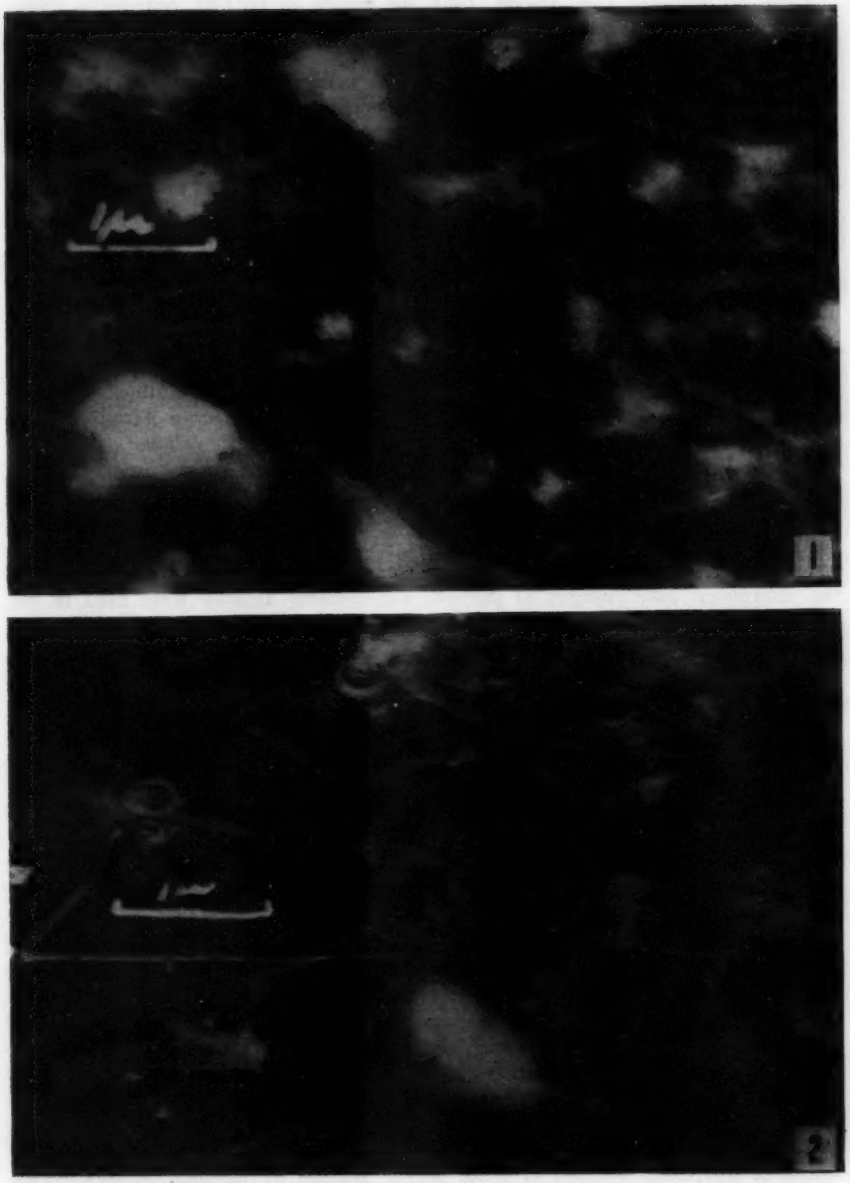
Living material and culture conditions

The following four species of Paramecium were examined in this study: *P. aurelia* (Sonneborn's stock 47, variety 4), *P. multimicronucleatum* (from Kirby's laboratory), *P. caudatum* (from Kirby's laboratory) and *P. calkinsi* (originally from Woodruff's collection). The first three of these were cultured in baked lettuce infusion medium (Sonneborn, 1950) which had previously been inoculated with *Aerobacter aerogenes*. *P. calkinsi* was cultured in identical fashion except that sea water diluted to 2/5 with distilled water was substituted for the distilled water and the $CaCO_3$ was omitted from Sonneborn's formula. With but one or two exceptions only cultures of starved, non-dividing animals were used in the study.

Preparation of the material for electron microscopy

As starting material for the preparation of pellicular fragments rich flourishing cultures of 100 to 300 ml. volume proved to be most satisfactory. When necessary, such cultures were filtered through a loose layer of cotton to clarify the suspension. The culture was then mixed rapidly with sufficient full strength formalin (40% formaldehyde) to give a final formalin concentration of 1 to 2%. The formalin-treated culture was allowed to stand for one to several hours during which time the fixed paramecia settled to the bottom of the vessel. At the end of this period of fixation, the supernatant was removed by aspiration and the concentrated sample of fixed animals was transferred to a 15-ml. centrifuge tube. The procedure employed here has been described in greater detail by Metz (1947) in an unrelated study.

Following fixation the paramecia were washed three times with distilled water (15-ml. washings with mild centrifugation) and finally suspended in 3-5 ml. of distilled water. To prepare the fragments of pellicle this final distilled water suspension was transferred to the receiving cup of a 9 Kc magnetostriction oscillator (Model S–102A, Raytheon Corp.) and vibrated for 30 seconds to several minutes. The duration of this treatment was governed by the degree of dissection desired and the fragility of the individual sample. Some variation was observed in the length of treatment required to break up the animals in different samples. This probably resulted from variations in the fixation procedure. To control these fac-



tors samples of the suspensions were removed from the receiving cup at 30-second to one-minute intervals and examined with the phase microscope. When the desired degree of disruption had been achieved, the treatment was terminated.

One- to 3-minute treatment usually resulted in thorough disruption of the paramecia. The cilia were stripped from the animals, the pellicles were broken into small fragments and the various formed elements of the endoplasm were released into suspension. Macronuclei, mitochondria and trichocysts in various stages of explosion were frequently observed. Since the present study concerned only the pellicular fragments and attached fibers, the other interesting structures were usually treated as undesirable contaminants and were separated from the pellicular fragments by differential centrifugation insofar as possible. At each step in the procedure the material was examined with the phase microscope. This was found to be necessary to prepare concentrated and relatively pure suspensions of fragments. Small drops of such suspensions were air-dried to collodion-coated electron microscope grids, shadow cast with palladium (*P. calkinsi, P. multimicronucleatum*) or chromium (*P. aurelia, P. caudatum*) and finally examined with the electron microscope (R.C.A. Universal).

Limitations

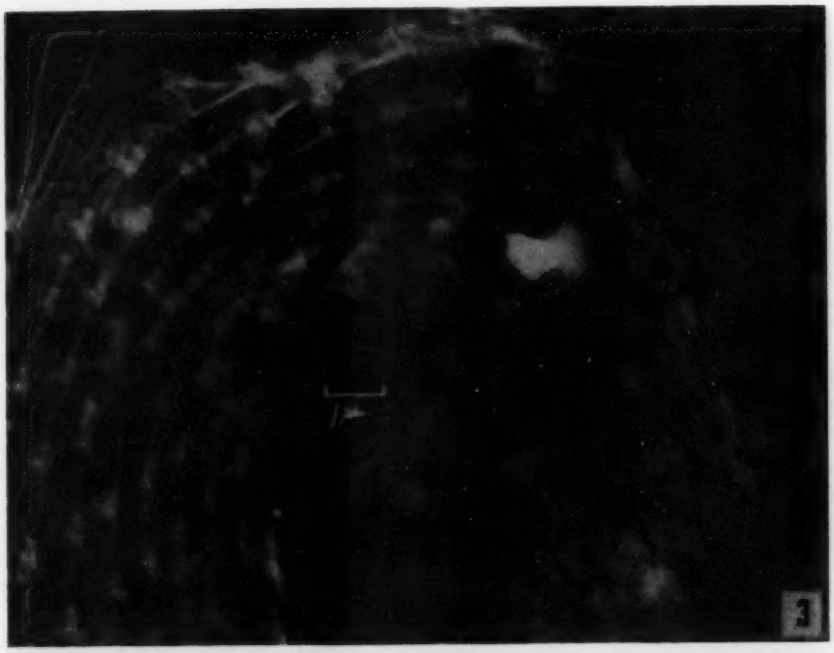
It is apparent that this method of preparation has certain inherent hazards and limitations. The first of these is the usual danger of introducing artifacts by fixation and drying. To minimize this possible source of error, the material was examined routinely with phase optics in the course of preparation and these observations overlapped those with the electron microscope. As a further precaution the fixation procedure was varied by using osmium tetroxide (osmic acid) vapor instead of formalin in a few instances. Within these limits no radical variation was observed in the preparations.

Sonic dissection as used here imposes further limitations on the study. Large numbers of animals were broken up together and fragments were selected for study on a chance basis. No systematic study of a single animal was attempted. To attempt to reconstruct a single organism by direct comparison of such fragments is hazardous and especially so since specific points of orientation are usually lacking in the fragments.

Finally, failure to find a particular structure or structures in these preparations cannot be considered immediate and clear proof of their non-existence in the intact organism, since such structures may have been removed or destroyed by the sonic treatment. The extraordinary feature of the preparations is that so many structures do remain intact.

FIGURE 1. *Paramecium aurelia.* A fragment of the pellicle viewed from outside, showing the polygon lattice in the form of hexagons and the subpellicular bundles of kinetodesmal fibrils. Approximately 21,600 ×.

FIGURE 2. *Paramecium aurelia.* A fragment of the pellicle viewed from inside the animal. Remnants of trichocysts are attached to the polygon cross bars (lower left). Single and double (right center) ciliary rings and accessory rings (upper left) appear in the centers of the polygons. Some of the ciliary rings are clearly plugged with cross sectional segments of cilia. Ciliary basal stumps and kinetosomes have disintegrated but the kinetodesmal fibrils remain intact. Approximately 24,000 ×.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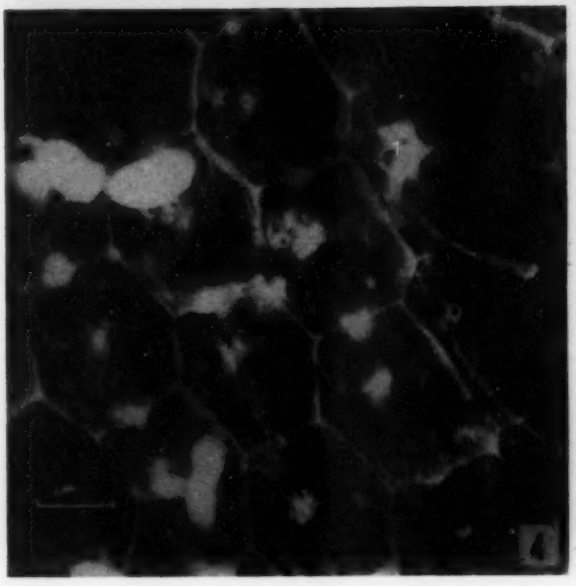
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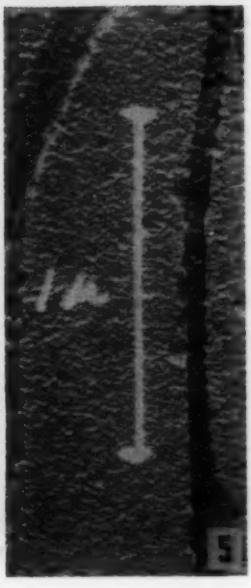
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The reported dimensions of structures and magnification scales must be considered as approximations, since the electron microscopes were not calibrated at regular intervals during the study.

Results

This descriptive account of the fibrillar systems in Paramecium is based largely on *P. aurelia* and *P. multimicronucleatum*. These two forms have been examined in greatest detail and the study is equally complete for both with the exception that no figures of the oral region of *P. multimicronucleatum* have yet been obtained. Since the two species were not found to differ, the account will apply to both forms.

Figures 1–4 show the type of specimen obtained when the material is properly treated with sonics. The first noticeable feature of the preparations is that the pellicular fragments are nearly free of cytoplasmic material. This might be expected since the pellicles of ciliates may be extracellular membranes (Beams and King, 1941). The really striking feature of the preparations is the collection of fibers that regularly remains attached to these pellicular fragments in spite of the sonic treatment. Indeed, with near minimal treatment, the cilia also remain attached (Figs. 6 and 7). Upon study of Figures 1, 2 and 4 it will be noted that the various structures in the specimens fall into two groups: A) the pellicle and an associated fiber-like lattice and B) a subpellicular fiber system. These are most conveniently considered separately.

A. The pellicle and pellicular lattice

As seen in Figures 2 and 4 the pellicle proper is a thin membrane. No ultrastructure is evident in this membrane at the magnifications employed. Intimately associated with the membrane is a fiber-like polygonal lattice. The polygons of this lattice usually assume the form of hexagons or quadrilaterals. In some preparations hexagons, quadrilaterals and intermediate forms are all present. With phase optics the polygon lattice appears as a system of ridges. The centers of the polygons are depressed. This fiber-like lattice is certainly the outer fibrillar system figured by numerous authors. The lattice appears as a continuous structure in all electron photomicrographs. No subfibrils or discontinuities are evident. Sedar (1952), however, reports that the material making up the lattice is compound in structure. The only modification in this structural uniformity is found on the cross bars of the lattice. At or near the middle of the cross bars, varying amounts of material remain attached (Figs. 1, 2, 4). In this region the lattice does not dissect clean. This position is described as the trichocyst attachment

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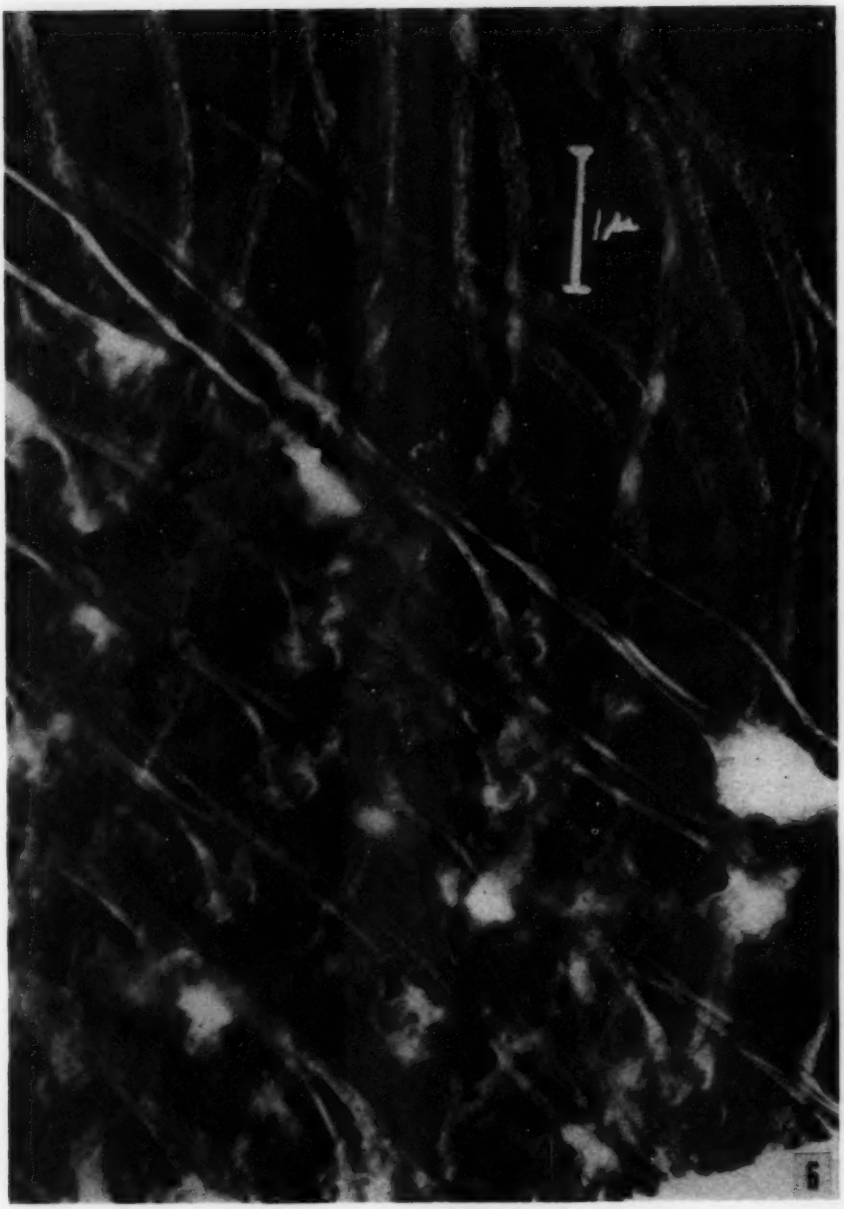
Figure 3. *Paramecium aurelia*. A circumoral fragment viewed from inside the animal. The ciliary rings and kinetosomes are paired. A single kinetodesmal fibril arises from each kinetosome pair. Fibrils run in an anterior direction. Those on the left side curve to the right in the preoral region. Approximately 9800 ×.

Figure 4. *Paramecium multimicronucleatum*. Fragment viewed from outside. Cones with pitted centers occur in the pellicle. These probably are the accessory rings (Fig. 2) seen from the outside. Several kinetodesmal fibrils are not anchored to kinetosomes, yet they remain attached to the kinetodesmal bundle. Approximately 12,000 ×.

Figure 5. *Paramecium calkinsi*. An individual kinetodesmal fibril showing spiral organization. Approximately 48,000 ×.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point by most earlier workers (*i.e.*, Lund, 1933; Sedar, 1952), and is so regarded here. However, it should be noted that in no case was an intact, undischarged trichocyst found attached to a pellicular fragment. In Figure 2 a rather substantial amount of material is attached to one cross bar of the lower left polygon. This may be part of an undischarged trichocyst. The suggestion of a fine fibrillar organization in the structure is of some interest.

The physical relationship between the outer lattice and the pellicle is disputed. Some investigators have held that the lattice is actually a thickened part of the membrane itself, whereas others (most recently Sedar, 1952) maintain that the membrane and lattice are separate structures. Unfortunately, this study does not clarify the issue materially. However, the lattice and the membrane appear to behave as a unit. No specimens were found in which the lattice and membrane were clearly separated. Furthermore, when the pellicle is torn the line of rupture frequently cuts across both membrane and lattice. Therefore, if these are separate structures rather than a continuous one, they must be cemented together rather firmly.

The cilium is generally stated to pass through the depressed center of the pellicular polygon. This view is in agreement with the present findings. Viewed from the inside of the pellicle (Figs. 6, 7) the cilium is clearly seen to pass through a ring-shaped structure. This is believed to be a thickening in the pellicular membrane. Occasionally (Fig. 2) two such rings occur in a single polygon. This situation appears to be constant in fragments from the circumoral area and agrees with the observations of J. von Gelei (1925, 1934a) that two cilia per polygon is a characteristic feature of this region in *P. nephridiatum* and *P. caudatum*. However, Lund (1933) finds only one cilium per polygon in the circumoral region of *P. multimicronucleatum*. It should be recalled that the oral region of *P. multimicronucleatum* was not examined in the present study.

In many preparations a second, smaller ring-shaped structure is found near the ciliary ring. This is seen most readily on an inside view of the pellicle (Fig. 2, upper left). The smaller ring, when present, is constant in position and occurs regularly in many if not all of the polygons in the specimen. The interpretation of outside views in terms of the ciliary ring and the accessory ring is more difficult. In Figure 4 interesting cones with pitted centers are evident. These could be either the external openings of the ciliary rings or the external parts of the secondary rings. The latter possibility is accepted because 1) these cones are very eccentric in position, 2) they do not appear to be associated with the kinetodesmal fibrils to be discussed in the next section, and 3) the opaque structures near the centers of the polygons correspond more nearly to the ciliary rings. These opaque structures are more central in position and larger than the cones; they are associated with kinetodesmal fibrils and finally they occasionally have a suggestion of a larger ring structure. The current investigation gives no hint of a possible function for these secondary rings.

One other structure associated with the pellicle is evident in some preparations. This is a strand or fiber extending across the polygon from one trichocyst attach-

FIGURE 6. *Paramecium aurelia*. Inside view of fragment. Cilia are seen to pass through the ciliary rings in the pellicle and end at the kinetosomes. Kinetodesmal fibrils pass from the kinetosomes and join the kinetodesmal bundles. Approximately 21,000 ×.

ment point to the ciliary ring and on to the next trichocyst attachment point. Such structures are seen in Figures 1 and 2. The nature of these is not clear. They appear to be fibers of the same sort and continuous with the lattice fibers in Figure 2. However, they are not present in all preparations, particularly those subjected to prolonged sonic treatment, and their form is somewhat variable (Fig. 1). If this material should take a silver stain, it could well have been confused with the primary fibrillar system to be discussed next.

B. The primary fibrillar or kinety system

Although the pellicle and the outer lattice are not without interest, the primary system is the striking feature of this study. The essentials of this are clearly illustrated in Figure 6 and consist of the three following parts: 1) the cilium which passes from the exterior through the ciliary ring in the pellicle and extends a short distance into the interior, 2) a bulb or knob at the internal terminus of the cilium, and 3) a long tapering fibril that arises from this knob and joins others of the same sort to form a bundle. This bundle runs parallel to both the surface of the pellicle and the rows of cilia.

The cilium

The cilium itself requires little comment. Its structure, as observed here, agrees with the descriptions of other workers. Thus, the free shaft of the cilium is seen to contain a number of fine longitudinal fibrils and from the studies of Metz and Pitelka (Metz, 1953) it is known to possess a limiting membrane. Unfortunately no clear picture of the proximal origin of the intraciliary fibrils has been obtained in this study although there are indications (Fig. 7) that these fibrils extend to the basal knob. In Figure 2 some of the ciliary rings in the pellicle appear to contain structures that correspond to cross sectional views of the cilium with the contained fibrils. It should be noted that the cilium is never pulled out of the pellicle. When it is broken off, the break occurs at the pellicle. In some cases (Fig. 2) the cilium is broken on both sides of the pellicle. Nevertheless, the ciliary ring remains plugged with a cross sectional segment of cilium.

The kinetosome

In the opinion of the writers, the bulb or knob at the internal terminus of the cilium is the ciliary basal body or kinetosome. This structure has a diameter (*P. multimicronucleatum*) of approximately 0.27μ (the kinetosome is assumed to have suffered no appreciable flattening since it casts a respectable shadow). This value is about 2.5 times that obtained for the cilium base and the fibril base (0.1 and 0.12μ in diameter, respectively; calculated on the assumption that these structures are flattened cylinders in the photomicrographs). Comparable values were obtained in *P. aurelia*. When two ciliary rings occur in one polygon, two corre-

FIGURE 7. *Paramecium aurelia*. A segment of the two middle kineties of Figure 6. Approximately 60,800 ×.

FIGURE 8. *P. multimicronucleatum*. A single kinetodesmal bundle extending beyond the torn pellicle. Terminal portions of five component fibrils are shown. These fibrils are seen to overlap in shingle-like fashion. Approximately 15,400 ×.

sponding kinetosomes appear. This is most evident in the oral region (Fig. 3).

In some figures (*i.e.*, 6, 7) the kinetosome seems to be grooved in the direction of the cilium. Otherwise there is no indication of internal structure or multiple organization. No accessory kinetosomes to correspond to Nebenkörner (Klein, 1928; von Gelei, 1932) occur in any of the figures.

The kinetodesma

Studies with the light microscope show that the kinetosomes are connected by a longitudinal fiber, the kinetodesma or neuromotor fiber. The electron microscope resolves this into a bundle of fibrils (especially clear in Figs. 6 and 7). Each of the component fibrils in the kinetodesmal bundle arises independently from a kinetosome, passes to one side and joins the main bundle. The kinetodesmal fibrils are not of indefinite length and constant diameter, but taper gradually to end in the main bundle. Thus, the main kinetodesmal bundle consists of a number of fibrils that overlap in shingle-like fashion along the bundle. This is most clearly seen in Figure 8. Here a bundle extends beyond the torn edge of the pellicle. The terminal portions of five component fibrils are clearly shown in this figure. The period between the ends of adjacent fibrils averages approximately 2.3μ. This value compares favorably with the interkinetosome interval (2.0 μ) calculated from another specimen. Evidently then, the kinetodesma of the light microscopist actually consists of a number of fibrils. Each of these arises independently from a kinetosome, joins the bundle and gradually tapers to a fine point. Apparently the kinetodesmal fibrils are of constant length, at least in a limited segment of the bundle. When two ciliary rings with the corresponding kinetosomes occur in a single polygon, both kinetosomes are joined to a single kinetodesmal fibril. This is particularly evident in the oral area.

To judge from the position of the trichocyst attachment points in the outer lattice and the arrangement of the polygons in the pellicular fragments, the kinetodesmal bundles must assume an approximately longitudinal course, with respect to the axis of the animal, except in the oral region. This agrees with the findings of earlier workers (*i.e.*, Lieberman, 1929). Not only do the bundles run longitudinally but the component fibrils are definitely polarized with respect to the axis of the animal. All of the fibrils join the bundle and pass along it in one direction. This is clearly seen in Figures 2, 4 and 6 and is true of every specimen examined that did not clearly represent part of the oral region. In the absence of any anterior or posterior reference point in the fragments it is not immediately clear whether the fibrils run from a posterior to a more anterior region or the reverse. But assuming that the "rule of desmodexy" (Lwoff, 1950) applies in Paramecium, it can be stated with some degree of confidence that the fibrils proceed in an anterior direction from the kinetosomes. According to the rule of desmodexy the kinetosomes lie to the right of the corresponding kinetodesma as the ciliate is viewed in normal orientation. Inspection of the figures shows that the kinetodesmal bundles lie to one side of the kinetosomes and that the individual fibrils curve laterally from their kinetosomal origins to join the main bundle. This condition is probably exaggerated somewhat by the action of surface tension when the preparation is dried. In this connection it should be noted that the kinetodesma appears as a line directly connecting adjacent kinetosomes in most light microscope studies.

In any event, the kinetodesma would necessarily have to deviate sufficiently from a straight course to pass around the trichocyst. Most of the figures presented here are so oriented that the kinetosomes lie to the right of the main kinetodesmal bundle as the fragment is viewed from its outer surface in accordance with desmodexy. In some of the figures (2, 3, 6 and 7) the pellicle is viewed from inside the animal. In these the kinetosomes will appear to the left of the kinetodesmal bundle. When oriented in this way it is apparent that the fibrils pass upward. Thus, if desmodexy applies in Paramecium, it is evident that the fibrils pass in an anterior direction from the kinetosomes.

The arrangement of the circumoral kinetodesmas adds support to this view. At least in the *aurelia-caudatum-multimicronucleatum* group of paramecia the kinetodesmas on the left ventral side of the animal (as viewed from the dorsal surface) pass forward, curve around the anterior margin of the oral opening and finally terminate in the preoral suture (Lieberman, 1929). The kinetodesmas on the right ventral side remain more nearly parallel to the suture. Therefore, the system in the oral region is asymmetrical and an isolated oral fragment can be oriented with respect to its position in the animals. When this is done (Fig. 3) all the kinetodesmal fibrils, on both the left and the right sides of the oral opening, are seen to pass in an anterior direction from their kinetosomes.

Structure of the individual kinetodesmal fibrils

No special effort was made in this study to investigate the structure of the individual kinetodesmal fibrils. Nevertheless, certain interesting features are evident in the preparations. These tapering fibrils show a periodic structure when shadowed longitudinally. In the best figures (*P. calkinsi*, Fig. 5) this takes the form of a spiral with a calculated period of approximately 400 Å. The spiral organization extends from the base to the tip of the fibril and its period appears to remain constant throughout the length of the fibril in spite of the fact that the fibril tapers to a point from a base diameter of approximately 0.12 μ (*P. multimicronucleatum*). It should be noted that the electron photomicrographs reveal this structure as a spiral ridge in the surface of the fibril. This ridge resembles the threads of a screw and casts the shadows which resolve the spiral in the photomicrographs. In order to preserve definition Figure 5 was not "reversed." Therefore, shadows appear light against a dark background in this figure.

The fibril never frays or breaks down in the preparations. It usually remains intact throughout its length in *P. aurelia, P. caudatum, P. multimicronucleatum,* but breaks off rather frequently in *P. calkinsi.* The fibrils themselves appear to be the most resistant parts of the system. They sometimes break free at their bases from their kinetosomes (Fig. 4) or the kinetosomes and the proximal stumps of the cilia may disintegrate (Fig. 2), but the fibrils remain intact and retain their characteristic organization. A thorough study of these extraordinary structures should prove very interesting. Perhaps more details could be resolved by partial digestion with enzymes or by examining sections at high magnification.

It is not clear how the overlapping fibrils are held together to form the kinetodesmal bundles. In some specimens the individual fibrils appear to be tightly bound together (Figs. 1, 3), but in others the fibrils are separated to a greater or less degree (Figs. 2, 6, 7, 8). Occasionally they even appear as completely iso-

lated structures. Presumably the tightly bound condition is the natural one. The binding agency must involve strong forces, for in many of the preparations (see Fig. 4) a number of fibrils are free from their kinetosomal anchors but nevertheless remain bound to the bundle in normal orientation. No evidence for a sheath about the bundle or any appreciable cementing material between individual fibrils has been found to account for this.

Commissural connectives

Some previous investigators have described cross connecting or commissural fibers extending laterally to adjacent kinetodesma. Lund (1933) figures these as connecting kinetosomes and states that they occur occasionally in the general body fibrillar system and that they form a complex lattice in the oral region. In the present study no evidence for such commissural connectives was found outside the oral area. Here, however, individual kinetodesmal fibrils sometimes pass at right angles to one another (Fig. 3). These may represent the commissural connectives of Lund.

Discussion

Studies on protozoan fibrillar systems are pertinent to several fields of interest. They contribute to the knowledge of protozoan morphology; they apply to the physiological problems of conduction, coordination and contraction; and they concern problems of morphogenesis. These aspects of the present investigation are considered below.

Morphological considerations

The main features of the pellicular lattice and kinetodesmal system of Paramecium, as seen in this study, agree with the findings of most previous workers. Indeed they confirm the views of J. von Gelei (1925), Lund (1933) and Worley (1933) in a most striking manner. The outer lattice is quite evidently distinct and anatomically separate from the primary or inner system. The only possible exception to this could be a connection between the trichocyst attachment point and the cilium ring in the pellicle. No evidence for any direct connection between the trichocysts and the kinetodesmal system was found. The study does not support Klein's (1926) concept of an indirect connection between the lattice and kinetodesmal system.

Certain other fibrillar structures have been described in Paramecium. Rees (1922) reported a system in which fibers arise from the kinetosomes, pass into the endoplasm and then proceed more or less directly to a neuromotorium located in the vicinity of the cytopharynx. No suggestion of the fibers of Rees was found in the electron photomicrographs. G. von Gelei (1937) has described still another fiber system. This constitutes a lattice at the level of the kinetosomes which ramifies throughout the animal at this level. No evidence for this system appeared in the current investigation. Although this study does not confirm the existence of these structures, it cannot be considered as critical evidence for their absence in view of the method of preparation of the material (see section on methods).

A number of investigators have described the kinetosome region of Paramecium as a compound structure (Klein, 1928; J. von Gelei, 1932; Sedar, 1952) containing

two or more distinct granules. One of these is usually considered to be a true ciliary basal body; the remainder as accessory bodies or Nebenkörner. In three of the four species examined with the electron microscope the kinetosomes appear to be single structures. In the exceptional form, *P. calkinsi*, two bodies which may be kinetosomes are frequently associated with each pellicular polygon. The study of this form is not sufficiently complete to determine whether this condition is general over the body surface or whether it applies only to the oral region as in *P. aurelia*. Likewise the detailed relationship of these bodies to the kinetodesmal fibrils in *P. calkinsi* has not yet been worked out.

Possibly compound kinetosomes may yet be found in *P. aurelia*, *P. multimicronucleatum* and *P. caudatum* after sonic treatment if the fixation procedure is varied. But whether or not such accessory structures are found, there is no question that at least one such structure joins the base of the cilium to the kinetodesmal fibril and that bundles of these tapering fibrils overlap to form the kinetodesma of the light microscopist.

Concerning this relationship of kinetosome to the cilium and kinetodesma, Worley's (1933) Figure 2 is of interest. This figure, a photomicrograph of a fresh preparation, clearly shows a series of bodies from which short fibers arise. These fibers appear to overlap. Worley makes no comment concerning this overlapping condition although he does state that direct connecting fibers (kinetodesma) are visible in the figure. However, it appears likely that these fibers are cilia which all lie in one direction.

Physiology

Nearly every student of Paramecium fibrillar systems has attributed specific functions to the various fibrillar structures. These functions include physical support, contraction, specific metabolic activity and various aspects of ciliary coordination. Unfortunately, few investigators have made any experimental attempt to support their theses, but rather follow the procedure of Lund (1933) who, on the basis of morphology alone, concludes (p. 58) that a correlating function "is almost to be accepted without choice" in the case of the kinetodesma. The most serious attempt to investigate the function of fibrillar systems in Paramecium by experimental means appears to be the study of Worley (1934).

Employing microdissection methods, this investigator made short transverse cuts through the ectoplasm of Paramecium and noted that the metachronal waves of ciliary action did not proceed beyond the cut. Metachronism was not disturbed elsewhere. Evidently, control of metachronism resides in the ectoplasm and the control follows longitudinal paths. In Worley's experiments ciliary reversal occurred simultaneously over the entire body surface and on both sides of ectoplasmic cuts. This suggests that the kinetodesmal fibrils do not control ciliary reversal. Although this evidence is neither extensive nor overwhelming, it does support the view that the kinetodesmal system has a role in ciliary coordination. For more extensive treatment of this aspect of the subject the reader should consult Parker (1929), Taylor (1941), Prosser *et al.* (1950) and Wichterman (1953).

The present demonstration that the kinetodesmas are not single fibers but consist of bundles of relatively short overlapping fibrils, each connected independently to a kinetosome, introduces a complication in any coordination theory of kineto-

desmal action. Coordination would have to pass laterally from one fibril to another— presumably to the fibril of the next adjacent cilium. This would imply a high degree of orientation of the fibrils with respect to each other and also that the relationship has the properties of a lateral synapse. In this connection it is of interest that *Tetrahymena* contains a specific acetylcholine esterase (Seaman and Houlihan, 1951) and that this enzyme appears to be confined to the ectoplasm of the ciliate (Seaman, 1951).

Many ciliates are highly contractile organisms. Although Paramecium is not notable in this regard, some investigators (Brown, 1930) have ascribed a contractile function to certain of the fibrillar elements. If Paramecium showed clear-cut contractile properties, it would be tempting to ascribe such action to the spiral organization of the kinetodesmal fibrils.

Morphogenesis

In ciliates the kinetosomes appear to be self-reproducing, genetically continuous entities; in certain forms they may act as pluripotent organization centers and finally they may be the seat of morphogenetic dominance and polarity (Lwoff, 1950; Weisz, 1951).

In Paramecium the demonstrated behavior and properties of the kinetosomes are somewhat less spectacular. During fission or as a preliminary to fission all body kinetosomes divide one or more times, the daughter granules separate along the longitudinal axis of the animal and finally form new cilia (von Gelei, 1934b; Downing, 1951). The kinetosomes of the oral region may be responsible for the specialized oral morphology and ciliature, since the new oral region is formed by a budding process from the original at fission (Hertwig, 1889; von Gelei, 1934b). The morphogenetic agents in the bud could be kinetosomes although evidence for this view is lacking. The origin of trichocysts is obscure (Wichterman, 1953); von Gelei (1934b) believes that new trichocyst granules arise from the neuronemes (kinetodesmas), a view which agrees roughly with that of Lwoff (1950) for apostomatous ciliates.

Although the present study was not designed specifically to clarify morphogenetic problems, it does re-define certain of these at a finer morphological level. This applies particularly to the problems of duplication and polarity of kineties. An individual kinety is seen to be a compound structure composed of definite units. Each unit is composed of a cilium, a kinetosome and a short, tapering kinetodesmal fibril. How, then, do these units duplicate and orient to form the compound structure of the kinety? According to current views the kinetosome is the primary duplicating organelle. By unknown mechanisms it can reproduce itself and it can produce other organelles. In keeping with this concept an individual kinetosome of Paramecium should be able to reproduce itself and produce a cilium with its complex internal structure. Ninety degrees from the site of cilium origin it should produce a kinetodesmal fibril with its taper and spiral symmetry. In the course of this study a single forked kinetodesmal fibril with two tapering ends was encountered. This unusual fibril could have arisen as a "developmental anomaly" from a kinetosome, but it is also possible that duplication is regularly initiated at the tip of the fibril and proceeds proximally to include the kinetosome.

In all of the preparations examined the kinotodesmal fibrils are polarized. They all taper in one direction and form tight bundles. If the kinetodesmal fibrils develop from the kinetosomes, "contact guidance forces" (Weiss, 1945) of some sort might cause a developing fibril to proceed along a pre-existing bundle, but such a concept does not so readily explain the exclusive anterior orientation in this polarity.

Refinement of the methods used here and their application to a variety of forms in stages of division and regeneration, in conjunction with sectioning techniques, may be expected to provide some insight into these problems.

The writers wish to express their great indebtedness to the late Professor Harold Kirby. With characteristic generosity Professor Kirby made his personal laboratory available to us for an investigation of Paramecium. It is a source of the deepest regret that Professor Kirby did not live to see the present study emerge.

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Summary

1. Various structures in Paramecium are readily obtained for electron microscope examination by formalin fixation followed by sonic dissection. These structures include macronuclei, mitochondria, trichocysts in various stages of explosion and fragments of the pellicle. This study concerns such fragments.

2. The electron microscope reveals two fiber systems associated with the pellicular fragments. One of these is intimately associated with the pellicular membrane and corresponds to the outer fibrillar lattice system of earlier investigators. The second system is subpellicular and corresponds to the kinety, neuroneme, or inner fiber system.

3. The pellicle proper consists of a thin membrane with no obvious fine structure. The outer lattice system is a continuous network. It corresponds in position to the system of polygonal ridges in the pellicle. A thickening in the cross bars of the polygonal lattice represents the trichocyst attachment point. A ring-shaped structure is found in the center of each polygon. The cilium passes through this ring. A short distance from this ciliary ring a second, smaller ring-shaped thickening is frequently found.

4. The kineties are compound structures composed of discrete units. Each unit consists of three parts: a) the cilium which passes through the ciliary ring of the pellicle and terminates internally at b), the ciliary basal body or kinetosome. Each kinetosome gives origin to c), a tapering fibril which parallels the surface of the animal. These units are associated by their tapering fibrils. The fibrils from a longitudinal row of kinetosomes overlap in shingle-like fashion to form a tight bundle. This bundle is the kinetodesma of the light microscopist. The fibrils of the kinetodesma all taper toward the anterior end of the animal.

5. No obvious connection exists between the outer lattice system and the kinety system.

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THE MECHANISM OF SYNERGISTIC ACTION OF DMC WITH DDT AGAINST RESISTANT HOUSE FLIES

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With the recent widespread occurrence of house fly resistance to many of the halogenated hydrocarbons, intensive efforts have been made to find chemicals which might enhance the effectiveness of these insecticides, especially DDT, against resistant house flies.

In the course of searching for such materials, Perry and Hoskins (1950, 1951a) found that piperonyl cyclonene increased markedly the effectiveness of DDT against various strains of DDT-resistant house flies. Sumerford, Goette, Quarterman and Schenck (1951) reported on the potentiation of DDT against resistant house flies by several structurally related compounds including 1,1-bis-(*p*-chlorophenyl) methyl carbinol (DMC). In a later work, Sumerford, Fay, Goette and Allred (1951) discussed the results of preliminary screening of several other candidate synergists for DDT. More recently, March, Metcalf and Lewallen (1952) have added to this list several effective synergists for DDT and closely related compounds.

Although a substantial amount of data has been reported on the potentiation of various insecticides by so-called synergists, little attention has been given to the biochemical significance of this phenomenon.

Various explanations have been advanced regarding the mode of action of synergistic compounds. David and Bracey (1944) attributed the action of several synergists in pyrethrum fly sprays to a delay in the knockdown of test insects, resulting in longer flying periods and in accumulation of larger amounts of the toxicant. Parkin and Green (1944), on the other hand, showed that retardation of knockdown was not a factor in synergistic action. Dove (1947) concluded that pyrethrum residues are stabilized in thin films by certain synergists, particularly piperonyl butoxide. Munro (1942), Lindquist, Madden and Wilson (1947) and others suggested that pyrethrum synergists may assist the penetration of the toxicant through the insect cuticle. Page and Blackith (1949) proposed that a loose molecular complex is formed between pyrethrins and synergists, and that the presence of those synergists influences the orientation of the pyrethrin molecules at the nerve-sheath interface, resulting in a greater concentration of the toxicant at the site of action. The observations made by Wilson (1949) on the action of piperonyl butoxide and piperonyl cyclonene with pyrethrum on house flies led him to conclude that the synergists damage a detoxifying mechanism. The detailed studies of Chamberlain (1950) on the mode of action of piperonyl butoxide with pyrethrins indicated that the enzyme lipase, obtained from roach and house fly extracts, was, in part, responsible for the detoxification of pyrethrins, and that inclusion of piperonyl butoxide in the formulation produced some degree of inhibition

of the hydrolytic action of lipase. More recently, Perry and Hoskins (1950, 1951b) demonstrated that the synergistic action with DDT imparted by piperonyl cyclonene was not the result of increased permeability of the house fly cuticle but was largely due to inhibition of detoxification of DDT.

The purpose of this report is to give a detailed account of the effect of 1,1-bis-(*p*-chlorophenyl) methyl carbinol (DMC) as a synergist for DDT, and to show a quantitative relationship between synergistic action and inhibition of DDT detoxification in resistant house flies.

Materials and Methods

The following materials were used in this study:

*p,p'*DDT; 2,2-bis-(*p*-chlorophenyl)-1,1,1-trichloroethane, recrystallized twice from ethanol; m.p. 108° C.

*p,p'*DDE; 2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethylene, obtained by dehydrochlorination of DDT in alcoholic KOH solution; m.p. 88° C.

*p,p'*DMC; 1,1-bis-(*p*-chlorophenyl) methyl carbinol, prepared from the technical product by fractional distillation under vacuum, followed by recrystallization from petroleum ether; m.p. 67–68° C. (*cf.* Grummitt, Buck and Becker, 1945).

*p,p'*DME; 1,1-bis-(*p*-chlorophenyl) ethylene, prepared by dehydration of DMC in the presence of anhydrous copper sulfate. The crude product obtained was purified by vacuum distillation, followed by recrystallization from petroleum ether and from ethanol; m.p. 84° C. (*cf.* Grummitt, Buck and Becker, 1945).

Application of benzene solutions of the chemicals was made topically by means of a micro-loop. The calibrated [1] micro-loop gave an average volume delivery of 0.00065 ml. (0.65 $mm.^3$) with less than 5 per cent error. The desired dosages were obtained by proper dilution of the solutions. The strain of flies used in this work was a multiple-resistant strain obtained from a local dairy and designated as Roberds. In a typical experiment, groups of 50 to 100 adult female flies, three to four days old, were anesthetized with carbon dioxide and the desired dosage was applied topically to the ventral thoracic region of individual flies. Following exposure, the flies were placed in holding cages provided with food and water and were kept at 26° C. and 60–70 per cent relative humidity. Mortality counts were made 24 hours after exposure.

The procedure used for studying the degradation of DDT and DMC was as follows: At a chosen interval after exposure, the flies were thoroughly rinsed in three successive 20-ml. portions of n-hexane to remove adhering particles of the chemicals. They were then ground in the presence of anhydrous sodium sulfate and extracted with carbon tetrachloride by mechanical agitation for one hour, and then filtered off. The external and internal extractions were tested for DDT and DDT-metabolites by the procedure of Schechter, Soloway, Hayes and Haller (1945). The latter procedure was also used in the determination of DMC and related compounds. The method of computation for mixtures of DDT and DDE was described in an earlier paper (Perry and Hoskins, 1951b). Since the Schechter-Haller color complexes of DDE, DMC and the ethylene derivative of

[1] Data from the Communicable Disease Center, Technical Development Branch, Public Health Service, Summary of Activities No. 25, p. 170, 1951.

DMC, 1,1-bis-(*p*-chlorophenyl) ethylene (designated as DME for convenience) have very similar absorption spectra, it is necessary to separate these compounds before quantitative determinations of each in a mixed solution can be made. To accomplish the separation of DMC and DME from DDT and DDE, use was made of a sulfuric acid-Celite column similar to that described by Davidow (1950) for the isolation of DDT from fat. A column (2.5 × 25 cm.) fitted with a glass stopcock was packed to a depth of 10 cm. The various materials dissolved in carbon

TABLE I

Separation of DDT and DDE from DMC and DME by sulfuric acid-Celite column

Number	Materials partitioned (100 microgram quantities)	Per cent recovery in eluate			
		DDT	DDE	DMC	DME
1	DDT	93.9	—	—	—
2	DDE	—	102.6	—	—
3	DMC	—	—	None	—
4	DME	—	—	—	None
5	DDT DMC	85.4	—	None	—
6	DDT DME	95.6	—	—	None
7	DDE DMC	—	96.3	None	—
8	DDT DMC DDE	96.5	96.1	None	—
9	DDT DME DDE	94.3	99.8	—	None
10	DDT DMC DME	87.1	—	None	None

tetrachloride were added in 100-microgram quantities to the columns and eluted with carbon tetrachloride until 100 ml. of eluate had been collected. It was found that DMC and DME, when chromatographed singly or in combination with DDT and DDE, were retained by the column (Table I). However, no satisfactory method was found for recovering quantitatively the DMC and DME retained by the acid-Celite mixture.

In separating the various materials from fly tissues the extracts were evaporated, redissolved in 20 ml. of carbon tetrachloride and filtered through the column.

Results

The range of mortalities of the Roberds strain resulting from topical applications of benzene solutions of *p,p'*DDT alone or in combination with *p,p'*DMC are shown in Table II. For convenience in comparing the effectiveness of the many combinations used, the data are plotted in Figure 1 as log-dosage of DDT in micrograms per fly versus mortality on the probit scale. The dosages of DMC in micrograms per fly are given on each regression line. These data permit determination of the amount of DDT required for any level of mortality with a given dosage of the synergist.

The synergistic effect resulting from separate applications of DDT and DMC was determined by pre-treating resistant flies with DMC at 6- and 24-hour in-

Table II

Twenty-four-hour mortality (per cent) of adult female house flies (Roberds strain) resulting from topical application of DDT and DMC at various ratios. Control tests showed no mortality

Micrograms/fly		Per cent mortality	
DDT	DMC	Range	Average
0.32	0.65	2–14	8
	3.25	10–25	17
	6.50	20–30	25
0.65	0.06	2–20	12
	0.32	15–50	31
	0.65	40–72	53
	3.25	72–98	85
	6.50	92–98	95
1.62	0.06	10–22	17
	0.32	44–88	54
	0.65	78–98	87
	3.25	100	100
3.25	0.06	26–68	41
	0.32	68–96	86
	0.65	100	100
6.50	0.06	42–58	50
	0.32	90–96	93
	0.65	100	100
13.0	0.06	61–75	68
6.5	—	8–30	15
13.0	—	24–62	40
26.0	—	46–75	67
52.0	—	72–98	88
—	6.50	0	0
—	13.0	0	0

tervals before treatment with DDT. The reverse procedure, *i.e.*, application of DDT followed by DMC at the same intervals was also used. Simultaneous applications of DDT and synergist were used for comparison. In each case mortality counts were made 24 hours after application of the second compound. The results (Table III) indicate a marked decrease in mortality when applications of DDT and DMC were separated by 6 and 24 hours, irrespective of the order of application and as long as the dosage of DDT was kept low. With the higher dosages of DDT it is evident that application of DDT followed by DMC at the indicated intervals was more effective than the reverse procedure. This may be explained on the as-

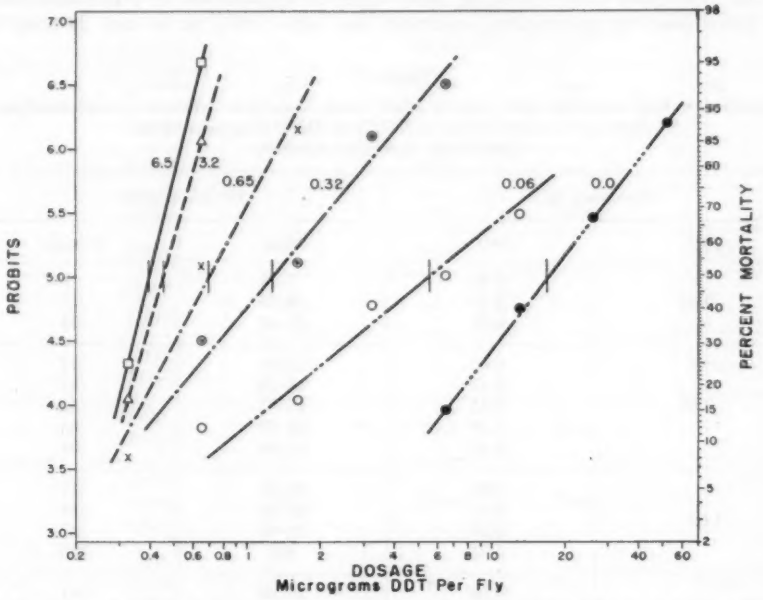


FIGURE 1. Probit-log dosage lines for adult female house flies (Roberds strain); DMC in micrograms per fly as indicated on the curves, plus various amounts of DDT applied topically to the ventral thoracic region.

sumption that sufficient unchanged DDT was still present when the DMC was applied, especially at the 6-hour interval, to produce high synergistic activity. Since in all cases simultaneous applications of DDT and DMC showed greater activity than separate applications of the chemicals, an explanation of the cause of synergistic action was sought in changes undergone by DMC after absorption.

To test this hypothesis, groups of flies were given topical applications of DMC in the usual manner. At various intervals ranging from 2 to 72 hours after exposure the flies were sacrificed and analyses of external and internal DMC were made by the procedure described earlier. Table IV shows that 24 hours after application of 13.0 micrograms DMC per fly, external rinsing removed approximately

TABLE III

Dosage-mortality data for simultaneous and separate applications of DDT and DMC at various intervals. DDT at 6.50 micrograms per fly gave an average mortality of 12 per cent. DMC had no effect at highest dosage shown

Material and dosage in micrograms/fly			Per cent mortality			
			DDT followed by DMC		DMC followed by DDT	
DDT	DMC	DDT:DMC mixture	Hours			
			6	24	6	24
0.32	3.25	26	0	0	12	2
0.65	0.65	48	0	0	6	2
0.65	3.25	82	8	0	60	4
3.25	0.32	89	80	16	24	0
6.50	0.32	96	90	58	64	10

0.4 micrograms DMC and internal extraction yielded 1.1 micrograms, giving a total of 1.5 micrograms, equivalent to 11.5 per cent recovery. Hence the undetected material, equivalent to 88.5 per cent, was either excreted or changed to some other product which did not respond to the method of analysis.

The former assumption was tested by collecting the excreta from DMC-treated flies held with food and water in covered beakers. At the end of the holding period, usually 24 hours, the flies were sacrificed and analyzed in the usual manner. The excreta from the beakers, food containers and cheesecloth covers were thoroughly washed with several portions of 2 per cent aqueous solution of sodium hydroxide followed by several rinses with n-hexane. The solvent and alkali were combined and shaken for several minutes, after which they were centrifuged and the layers separated. Analysis of the hexane portion showed the presence of only small amounts of DMC (Table V). The alkali layer was therefore acidified to pH 2–3 with 6 N sulfuric acid and extracted with ether to determine if any

TABLE IV

Amounts of external and internal DMC recovered from adult female house flies (Roberds strain) at various intervals following topical application of 13.0 micrograms DMC per fly

Interval to extraction (hours)	DMC recovered				Per cent DMC unaccounted (by difference)
	External		Internal		
	μg./fly	Per cent	μg./fly	Per cent	
2	7.65	58.8	4.50	34.6	6.6
4	5.75	44.2	6.50	50.0	5.8
8	2.05	15.7	4.46	34.3	50.0
16	0.65	5.0	1.80	13.8	81.2
24	0.55	4.2	1.48	11.4	84.4
48	0.25	2.0	0.54	4.1	93.9
72	0.17	1.3	0.50	3.8	94.9

acidic derivatives of DMC might be found. Results of four to six tests, which were averaged for each dosage (Table V), indicated the presence in the excreta of a metabolic product of DMC tentatively identified as bis-(*p*-chlorophenyl) acetic acid (DDA), on the basis of its solubility in dilute alkali. Orienting tests showed that DMC and DME are not removed by dilute alkali. None of the above products was found in extracts from untreated flies which served as controls.

From chemical considerations it appears that the conversion of DMC to DDA must proceed through the mediation of some intermediate product(s). The ease with which DMC undergoes dehydration *in vitro* suggests that the first product of degradation might be the ethylene derivative, 1,1-bis-(*p*-chlorophenyl) ethylene (DME). Tests with DME, similar to those described above for DMC, also showed the presence in the excreta of the same product, DDA (Table V). Topical applications to resistant flies of DDT-DME combinations evinced some synergistic activity, although not as marked as that with DDT-DMC. This might

TABLE V

Determination of DMC, DME and their degradation products 24 hours following topical application of DMC or DME to adult female house flies. Average of four to six tests with each dosage

Material and dosage applied in micrograms per fly	Product recovered	From tissue extracts		From excreta	
		Micrograms per fly	% of amount applied	Micrograms per fly	% of amount applied
DMC 6.5	DMC and/or DME	0.69	10.7	0.32	4.8
	DDA	0.62	9.5	4.20	64.6
DMC 13.0	DMC and/or DME	1.62	12.5	0.66	5.0
	DDA	0.84	6.4	6.80	52.3
DME 13.0	DME	1.30	10.0	0.18	1.4
	DDA	0.70	5.4	6.50	50.0

also serve as an indication of the intermediate role played by DME in the metabolism of DMC. In addition to DME, it is conceivable that one or possibly more intermediate(s) are formed in the conversion of DMC to DDA.

The degradation of DDT to the relatively nontoxic derivative DDE has been shown by Sternburg, Kearns and Bruce (1950). Perry and Hoskins (1950, 1951b) and Fullmer and Hoskins (1951) further demonstrated the inhibiting action of piperonyl cyclonene on the degradation of DDT. Winteringham, Loveday and Harrison (1951) obtained the same results with the bromine analog of DDT. If ability of resistant flies to survive is, in part, a function of detoxification of DDT, then inhibition of this process should show a quantitative relationship to mortality.

To obtain quantitative data on this subject, a series of tests was designed to determine: (1) the amounts of DMC in combination with a fixed dosage of DDT required to produce varying levels of mortality; (2) the effect of these amounts of DMC upon the degradation of DDT; and (3) the correlation, if any, between inhibition of detoxification and mortality. The results of three tests with groups of

100 flies per test shown in Table VI make such a correlation possible. Per cent inhibition was calculated as follows:

$$\frac{\text{Amount of DDE recovered from application of DDT and DMC}}{\text{Amount of DDE recovered from application of DDT alone}} \times 100 = X$$

$100 - X =$ per cent inhibition.

It may be noted that as the dosage of DMC was increased there was a corresponding increase in per cent inhibition of DDE formation and, consequently, an increase in mortality. It is also evident that complete inhibition was not essential for 100 per cent mortality of resistant flies.

TABLE VI

The effect of DMC on inhibition of conversion of DDT to DDE and on mortality of adult female resistant house flies. Extractions made 24 hours after application. DMC separated chromatographically

Applied micrograms/fly		Recovered*				Per cent inhibition	Per cent mortality
		DDT		DDE**			
DDT	DMC	μg./fly	% of DDT applied	μg./fly	% of DDT applied		
0.65	0.0	0.0	0.0	0.51	78.4	—	0.0
0.65	0.06	0.06	9.4	0.41	63.0	19.6	2.0
0.65	0.13	0.11	16.9	0.38	58.4	25.5	14.0
0.65	0.32	0.18	27.7	0.32	49.2	37.2	25.0
0.65	0.65	0.29	44.6	0.26	40.0	49.0	50.0
0.65	1.30	0.35	53.8	0.18	27.7	64.7	72.0
0.65	3.25	0.45	69.2	0.12	18.4	76.5	92.0
0.65	6.50	0.50	76.9	0.08	12.3	84.3	100

* External rinses showed no DDT or DMC present.
** DDE figures are expressed as molecular equivalents of DDT.

DISCUSSION

Synergism has been defined in many different ways; however, most definitions agree on one common point, *i.e.*, that the physiological effect of the substances in a mixture is significantly greater when used together than the summation of their individual effects. Bliss (1939) suggests that when the constituents of a mixture act independently, its toxicity does not depend upon the relative proportions of the components but only upon their inherent potencies. Synergistic action, in contrast, involves the ratio of one component to the other.

A conspicuous feature of Figure 1 is that as increasing amounts of DMC are added to DDT the probit-log dosage lines move toward smaller dosages of DDT and, in addition, become successively steeper. This process continues until a point is reached beyond which further addition of the synergist, within limits, causes either no increase or a slight decrease in activity. As seen from Figure 2, the addition of a small amount of DMC caused a significant drop in the amount of DDT required for a given mortality, whereas with large dosages of the adjuvant the effect was much less pronounced, as indicated by the hyperbolic nature of the curves. This reduction in DDT was roughly proportional to the mol fraction of DMC over

a limited range of dosages, until the DMC was present in approximately equimolecular proportions with DDT. Figure 2 indicates that but little could be gained by further addition of DMC beyond this point; hence, by visual inspection, it may be reasonable to suppose that the 1:1 ratio is the optimum ratio of DDT:DMC.

It may also be noted (Fig. 1) from the data for the LD_{50} and the LD_{90} with DDT alone, and those for DDT plus the maximum amount of DMC, that the resistance of the Roberds strain may, under experimental laboratory conditions, be reduced to the extent of about 98 per cent (from 17.0 gamma to 0.40 gamma DDT per fly for the LD_{50}, and from 56.0 gamma to 0.58 gamma per fly for the LD_{90}). It should be borne in mind that these values are calculated in terms of amounts of

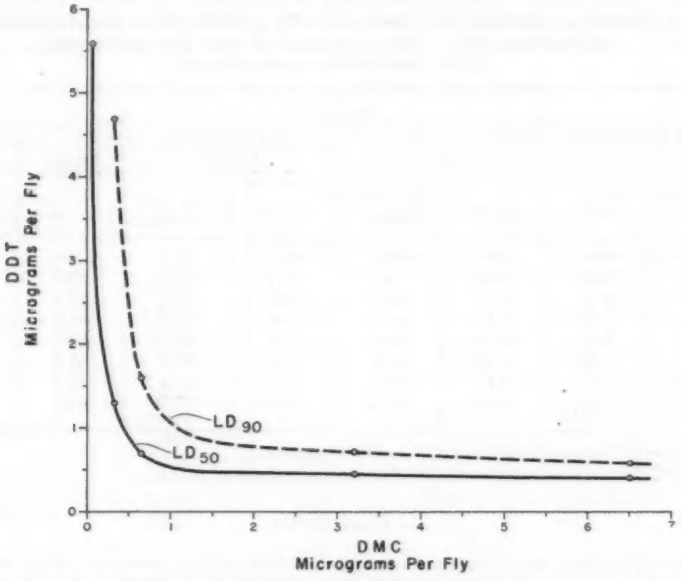


FIGURE 2. Curve relating dosage of DDT with dosage of DMC for 50 per cent and 90 per cent mortality of adult female house flies (Roberds strain).

DDT applied. Since the rate of absorption of DDT by house flies does not increase proportionally to increasing dosages by topical application (Fig. 3), the percentage reduction of resistance, if calculated on the basis of DDT absorbed, would be somewhat lower than 98 per cent. In no case, however, was it possible to reduce the resistance of the Roberds strain completely to the level of the susceptible strain (LD_{50} approximately 0.30 microgram per fly).

There is reason to believe that synergistic compounds do not all act in the same manner, nor do they affect the same physiological system *in vivo*. It has been shown by various workers (Lindquist, Madden and Wilson, 1947; Wilson, 1949; Chamberlain, 1950; Perry and Hoskins, 1951a; and others) that pre-treating house flies with synergists followed by application of pyrethrins or DDT at different

intervals did not materially affect the resultant mortalities as compared with simultaneous application of both compounds. In the present case, however, a marked reduction in activity resulted when applications of DMC and DDT were separated (Table III). This may indicate a different mechanism of synergistic action from those mentioned above.

The data of Tables IV and V show that DMC is rapidly metabolized by living flies. Thus, in 24 hours, from 50 to 70 per cent of the amount of DMC applied is excreted as DDA, and from 10 to 15 per cent is retained in the body either un-

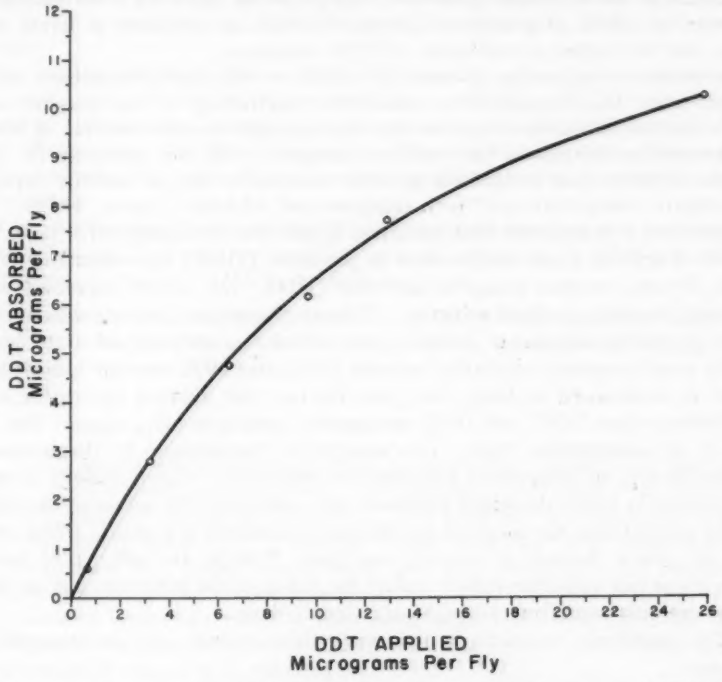


FIGURE 3. Curve relating amount of DDT absorbed in 24 hours with amount of DDT applied to adult female house flies (Roberds strain).

changed or in the form of DME. When sub-lethal amounts of DDT-DMC combinations are applied, both DDE and DDA are formed in substantial quantities. Lethal dosages of DDT-DMC, on the other hand, yield large amounts of DDT, small amounts of DDE and only traces of DDA. As a typical example, application of 0.3 gamma DDT and 0.3 gamma DMC per fly yielded in 24 hours 0.07 gamma DDT, 0.16 gamma DDE and 0.18 gamma DDA per fly, and the resulting mortality was 0–10 per cent. On the other hand, application of 0.6 gamma DDT plus 2.4 gamma DMC per fly yielded 0.36 gamma DDT, 0.13 gamma DDE and only traces of DDA, while the resulting mortality was 70–90 per cent.

Since no satisfactory analytical methods for distinguishing between DMC and

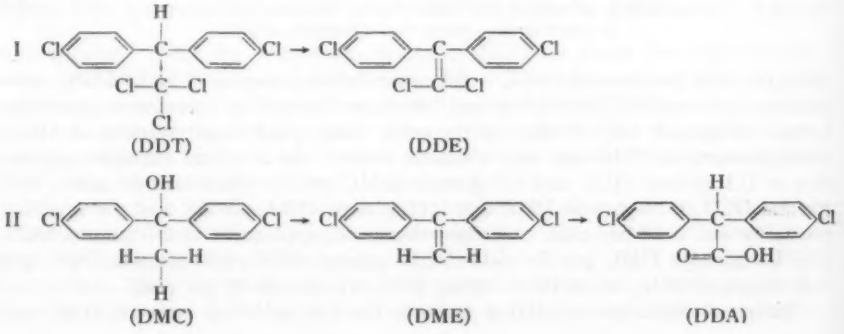
DME are available, the role of DME in intermediary metabolism cannot be ascertained. However, on the basis of the following considerations, namely, (a) the relative ease of dehydration of DMC *in vitro,* (b) the conversion of DME to DDA *in vivo,* and (c) the fact that DME exhibits some synergistic activity with DDT, it is postulated that DME might be an intermediate product of DMC metabolism.

In attempting to elucidate the mechanism of synergistic action of DMC it is assumed that dehydrohalogenation of DDT, dehydration of DMC, as well as formation of DDA are enzymatic processes. As shown in Table VI a correlation exists between the extent of synergistic activity of DMC, as measured in terms of mortality, and the degree of inhibition of DDE formation.

Inhibitors of enzymatic reactions are usually of two types, competitive and non-competitive. In non-competitive inhibition, inactivation of the enzyme is independent of substrate concentration and depends only on concentration of inhibitor. In competitive inhibition, the inhibitor competes with the substrate for specific groups of the enzyme so that the apparent decrease in enzyme activity depends on the relative concentrations of both substrate and inhibitor (Lardy, 1949). In the present case it is apparent that inhibition is not of a non-competitive type, for the degree of activity is not independent of substrate (DDT) concentration (Fig. 1). Thus, for any constant dosage of inhibitor (DMC) the activity increased with increasing amounts of added substrate. Likewise, increased activity was manifested when increasing amounts of inhibitor were added to a constant substrate dosage.

The great structural similarity between DMC and DDT and the knowledge that DMC is metabolized by living flies, plus the fact that greatest synergistic effect is manifested when DMC and DDT are applied simultaneously, suggest that inhibition is of a competitive type. This concept of "competition by displacement" is unlike the type of competitive inhibition by structurally related analogs of essential metabolites in which the added substrate may counteract the action of the inhibitor. In the present case the so-called metabolite or substrate is a poison which resistant flies are able to detoxify in varying quantities. Clearly, the addition of increasing amounts of this substrate cannot nullify the action of the inhibitor, but on the contrary, can only contribute to the injury already done.

The postulated reactions in this competitive system may be summarized as follows:



The quantitative relationship between inhibition of DDE formation and mortality of resistant house flies (Table VI) indicates that detoxification of DDT is a major factor in survival. If this were not the case, it would be difficult to explain why resistant flies should succumb to small quantities of DDT-DMC mixtures when they are able to tolerate large dosages of DDT.

DMC is non-toxic to house flies at fairly high dosages, and no observable symptoms are noted from application of DMC alone. Unless DMC causes the derangement of an unknown physiological process in the fly which might be associated with resistance, the above data point toward detoxification of DDT as a major factor in survival, and to inhibition of this process by DMC as a factor in mortality.

The nature and properties of the DDT-detoxifying mechanism have not yet been ascertained. However, the work of Sacktor (1951a, 1951b) on cytochrome oxidase in resistant and susceptible house flies suggests that this enzyme system might be associated with DDT-resistance. The relationship, if any, between cytochrome oxidase, DDT-detoxification and the inhibiting action of synergists has not been studied.

It is believed that the data presented in this report lend support to the hypothesis manifesting a specific competitive type of inhibition of DDT-detoxification as the mode of action of DMC as a DDT synergist.

The writers are indebted to Dr. R. W. Fay, Chief of the Insecticide Section, and to Dr. G. W. Pearce, Chief of the Chemistry Section, for many helpful suggestions and criticism throughout this study.

Summary

1. DMC has no insecticidal properties but markedly enhances the effectiveness of DDT against resistant house flies. The addition of a small amount of DMC causes a significant drop in the amount of DDT required for a given mortality.

2. Data interpolated from probit-log dosage lines for DDT alone and DDT plus DMC show that, under experimental laboratory conditions, the resistance of the Roberds strain can be markedly reduced.

3. Greatest synergistic effect is manifested when DMC and DDT are applied together, for separate application of the chemicals at 6-hour and 24-hour intervals shows a marked reduction in mortality.

4. DMC is rapidly metabolized by living flies and is excreted principally as a product tentatively identified as bis-(*p*-chlorophenyl) acetic acid (DDA). The compound 1,1-bis-(*p*-chlorophenyl) ethylene is suggested as being an intermediate product in DMC metabolism.

5. A correlation is shown between the extent of synergistic action and the degree of inhibition of DDT-detoxification in resistant flies.

6. Inhibition appears to be of a competitive type, the synergist competing with the insecticide for the mechanism of detoxification.

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RECORDINGS OF HIGH WING-STROKE AND THORACIC VIBRATION FREQUENCY IN SOME MIDGES

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The highest known wing-stroke frequencies of insects have been recorded in male specimens of the dipterous families Chironomidae and Ceratopogonidae (Heleidae), where values of 1000/sec. have been obtained (Sotavalta, 1947). These recordings were made by registering acoustically the pitch of the flight-tone emitted by these insects during free flight. Until now, very few recordings of high wing-stroke frequencies have been obtained by the aid of other methods. Boettiger and Furshpan (1952) have obtained a wing-stroke frequency of 500/sec. in a chironomid midge, using electrostatic methods combined with cathode-ray oscillograph.

That still higher values of wing-stroke frequency can be produced, at least under artificial conditions, appears from the experiments reported below. The frequency determinations were made by three methods. The pitch of the flight-tone in free flight was determined acoustically; the flight-tone in free flight was also recorded by means of a microphone and high fidelity tape recorder, and with an accurate frequency check of 1000/sec. from a beat-frequency oscillator, transposed to recording film by means of a double-beam cathode-ray oscillograph; the thoracic vibration during fixed flight was registered by means of a piezo-electrical crystal pick-up and similarly recorded on tape and film. Male specimens of a small green species of *Chironomus* (s.lat.) and of a tiny species of *Forcipomyia* (Ceratopogonidae) were used in the experiments.

Experiments on Chironomus: Flight-Tone and Thoracic Vibration Frequencies

In order to record the flight-tone in free flight, the specimen was allowed to fly unmounted in a small glass chamber attached to the front of the microphone. The insect was stimulated to flight by letting it respond phototactically to a bright lamp near the glass chamber, or by shaking the glass chamber. The maximum duration of flight in the chamber obtained in this way was about 7–8 seconds. The flight-tone frequency recorded was about 600–650/sec. (Fig. 1), with occasional higher and lower values. Acoustical determinations were made before the experiment by allowing the insect to fly in a test-tube, the mouth of which was pressed against the ear. The general range of the flight-tone was determined as $d^{2}\sharp$–e^{2} (622–659/sec.), and thus thoroughly agreed with the oscillographic recordings.

[1] This work was done while the author held a U. S. Government grant under Finnish Educational Exchange Program. The author wishes to express his gratitude to Prof. K. D. Roeder for valuable suggestions and assistance, and to Dr. Willis W. Wirth for the identification of the genus of *Forcipomyia*.

The thoracic vibrations were recorded in other specimens by mounting the insect on a stylus inserted in a crystal pickup (Roeder, 1951). Releasing the flight reflex by removing a platform from under the tarsi induced fixed flights for as long as 15 seconds, which could be recorded.

The thoracic vibration frequency was found to be about 520–580/sec. (Fig. 2a). A loudspeaker was turned on during the experiments, and it emitted a loud tone which thus was produced not by the wings but by the *thorax*. The frequency of

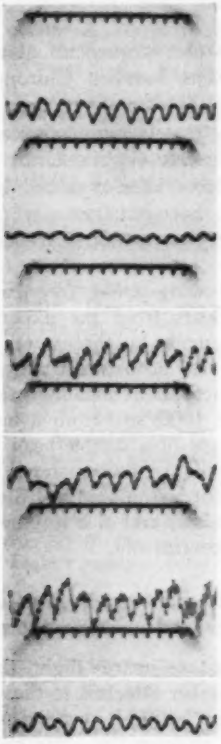

FIGURE 1. Flight-tone of *Chironomus* sp. and time-check 1000/sec. Frequency 600–680/sec.

this tone was determined acoustically as 554–587/sec. At the same time also the flight-tone produced by the wings was checked acoustically, and it was found in this case to have a pitch of $c^{2\#}$–d^2 (554–587/sec.), thus identical with the other determinations above.

The wings were then mutilated with transverse cuts, and a recording taken after each cut. First the left wing was cut to about half of its length. The thoracic vibration frequency (Fig. 2b), the frequency of the loudspeaker tone and of the flight-tone were again found to agree completely, being about 650–700/sec. (e^2–f^2).

The right wing was then cut to the equal half-length, and the frequency rose to about 830–880/sec. (g^2–a^2) (Fig. 2c). Both wings were then cut close to their bases, and the increased frequency was found to be about 1300–1400/sec. (e^3–f^3) (Fig. 2d).

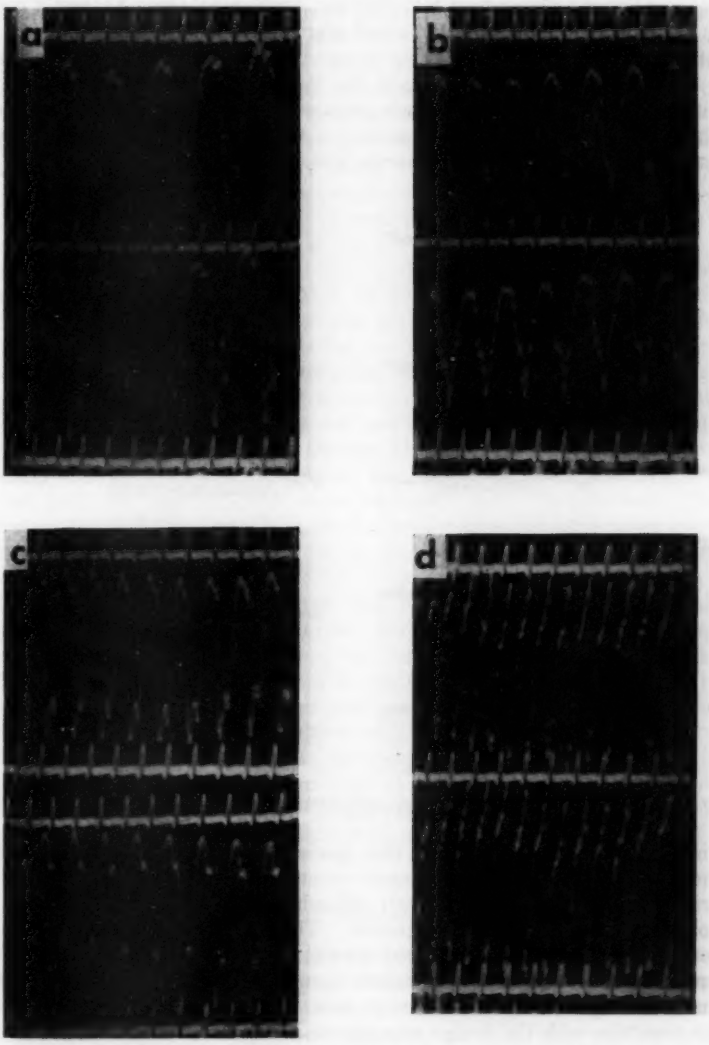

FIGURE 2. Thoracic vibrations of *Chironomus* sp. and time-check 1000/sec. (a) of an intact specimen, (b), (c) and (d) after three successive wing mutilations.

Experiments on Forcipomyia: Flight-Tone Frequencies

The specimen was allowed to fly unmounted in a glass chamber attached at the front of the microphone and flight was induced as in *Chironomus*. Also the flight-tone was checked acoustically before the experiment as above. The flight-tone frequency was found to be about 800–950/sec. ($g^2\sharp$–$b^2\flat$) (Fig. 3), and a complete agreement between the oscillographic and acoustic recordings was observed.

Unfortunately, it was not possible to mount a specimen of this species on the stylus, because even a slight heating of the mounting wax either killed the insect or, as it seemed, paralyzed the thoracic muscles. Any glue used for mounting did not adhere to the smooth and shiny surface of the thorax. Therefore no recording of the thoracic vibrations of these insects was possible.

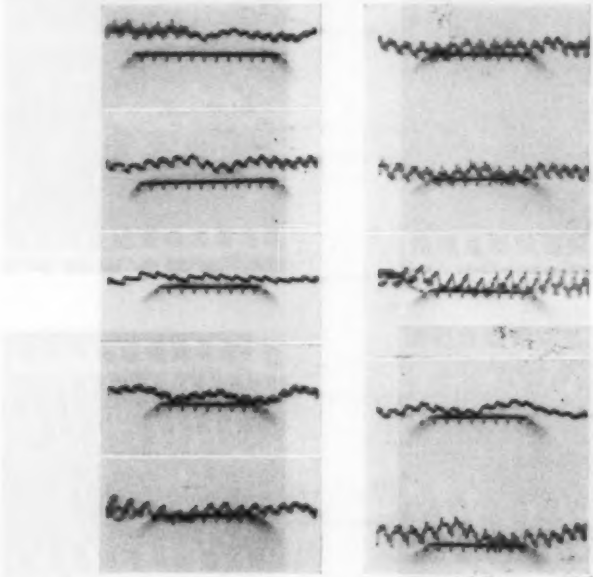

Figure 3. Flight-tone of *Forcipomyia* sp. and time-check 1000/sec. Frequency 800–950/sec.

Wing-mutilation experiments in this species were carried out with unmounted specimens. Since cutting the still more minute wings of such minute insects even with iridectomy scissors was extremely difficult without damaging the insect otherwise, only one experiment was successful. The wings were removed close to the base and the tone emitted determined acoustically by allowing the insect to "fly" in a small test-tube in front of an electric lamp. The flight-tone, which in the intact insect was $g^2\sharp$–a^2 (831–880/sec.), rose to f^3–$f^3\sharp$ (1397–1480/sec.). After this the test-tube with the midge was placed in an incubator at 37° C. for about 20–30 seconds. When it was taken out, the flight-tone was immediately determined. It proved to be a^3–$c^4\sharp$ (1760–2218/sec.). The midge, unfortunately,

died so quickly after this experiment that no tape or film recording of these high frequencies was possible.

Discussion

In order to produce a wing-stroke frequency of 1000/sec. the indirect flight muscles have to perform their complete cycle of contraction and relaxation in one msec. If the frequency is 2200/sec., this time is 0.45 msec., and thus both sets of indirect flight muscles perform 2200 contractions and relaxations during one second. Though it certainly is very doubtful whether there exist in nature insects which in an intact state possess a wing-stroke frequency comparable to the latter value, the producing of it artifically shows that the flight muscles are *capable* of such extreme performances under appropriate conditions. This fact also provokes a question about the minimum limit of time interval after which a muscle in general is able to repeat its contraction. Since the contraction frequency must be correlated with the intrinsic speed of the muscle, *i.e.*, with the ratio of contraction speed at zero load to fiber length (Hill, 1949), it follows that this limit, in turn, is correlated with the minimum limit of size in animals having well developed muscular organization. The problem whether motor nerves can transmit separate impulses with such a frequency is excluded because the indirect flight muscles of Diptera and of certain other insect orders possess a unique ability to maintain a rhythm of contractions without a motor nerve impulse preceding each contraction, as shown by Pringle (1949) and Roeder (1951). No microscopical insects, in the proper sense of the word, are known; therefore it is evident that in approaching this size limit the limit of contraction frequency also is approached. Since the speed of contraction also depends on load, even the most minute known insects cannot have an extremely high frequency of wing-strokes unless also the *relative* size of their wings is very small.

Since the energy output of a large and of a small insect is proportional to the cube of their linear dimensions, while the energy requirement is proportional to the fifth power of their linear dimensions, this leaves a margin in small insects within which the wing-stroke frequency can be increased (Sotavalta, 1952). Therefore a minute size is a *conditio sine qua non* for an extremely high wing-stroke frequency. In spite of this, however, there most likely appear additional factors which have a relatively greater significance as energy consumers in small than in large insects. The effect of drag in propulsion (*cf.* Sotavalta, 1952) and the dissipation of heat from the body to the air are proportional to the surface and therefore may necessitate a higher energy consumption in a small insect than predicted by the above reasoning.

Summary

1. Experiments on recording flight-tones and thoracic vibrations in intact and wing-mutilated midges (*Chironomus, Forcipomyia*), using double-beam cathode-ray oscillograph and film and acoustic method, are reported.

2. In *Forcipomyia,* a wing-stroke frequency of 2218/sec. was the maximum value recorded, produced in a specimen with wings cut and exposed to high temperature.

3. Some aspects of the occurrence of such high frequencies in insects, correlated to the muscle contraction frequency in general, and of the energy consumption in small insects are discussed.

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A COMPARATIVE STUDY OF THE RESPIRATORY METABOLISM OF EXCISED BRAIN TISSUE OF MARINE TELEOSTS [1]

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In recent years several investigators have studied the respiratory metabolism of fish brain tissue. Fuhrman *et al.* (1944) studied the metabolism of excised brain of the large-mouthed bass, *Huro salmoides,* at graded temperature levels. In 1950 the phenomena of brain tissue metabolic adaptation to temperature in the polar cod, *Boreogadus saida,* and the golden orfe, *Idus melanotus,* were investigated by Peiss and Field. Freeman (1950), working with the goldfish, *Carassius auratus,* noted their brain metabolism during temperature acclimatization.

Certain physiological factors have been correlated with activity in marine fishes. Dawson (1933) showed that the more active fishes had a greater number of circulating immature erythrocytes than did less active species. Hall (1929, 1930) found that sluggish species not only consumed less oxygen but were capable of removing oxygen to lower tensions than could more active fishes. Hall and Gray (1929) and Gray and Hall (1930) found that in general the more active surface-feeding fishes had higher hemoglobin concentration and higher blood sugar level than did the sluggish bottom dwellers. Gray (1946, 1947) has pointed out a direct correlation between activity and the area of gill surface in marine teleosts.

It has seemed worthwhile to make a comparative study of the respiratory rate of excised brain tissue of marine teleosts to see if there might not be a correlation between brain metabolism and activity.

Materials and Methods

Fishes were collected in the vicinity of the Duke University Marine Laboratory, Beaufort, N. C., and were maintained in the laboratory in aerated tanks supplied with running sea water. The procedures apply to all 17 species of marine fishes used in this study. Following exposure by cutting through the roof of the skull, all brain tissue anterior to the vagal lobes was removed, blotted quickly on filter paper, and weighed. The brain tissue was then ground in a dry mortar and taken up in a phosphate buffer of pH 7.5 (glass electrode) prepared by mixing 0.16 *M* KH_2PO_4 and 0.121 *M* $NaPHO_4$. Sufficient buffer was added to bring the volume to 3.0 ml. and then the brei was transferred to a Warburg flask. The center wall of the respirometer flask contained both 0.2 ml. of 10% KOH and filter paper wicks. Time between the death of the animal and the beginning of the 10-minute period of thermal equilibration was kept constant at 10 minutes. Manometric measurements were made in a bath maintained at 30° C. Readings were taken at 10-minute intervals and were carried out for a minimum of 60 minutes. Results

[1] Aided by a grant from the Duke University Research Council.

are expressed in terms of wet weight Q_{O_2}. Thus Q_{O_2} (wet weight) denotes microliters of oxygen consumed per gram of wet weight per minute. The water content of the brain tissue of some species was determined by drying to a constant weight at 105° C.

Results

The rate of oxygen consumption of brain brei preparations of 17 species of marine teleost fishes determined at 30° C. is shown in Table I. The fishes are arranged in the table, not in the order of their activity, but according to their rate of brain tissue oxygen consumption. Although a sharp dividing line is not evi-

Table I

Oxygen consumption of brain breis of some marine teleost fishes

Species	No. of determinations	Mean Q_{O_2} wet weight	Standard deviation
Group I:			
Menhaden *Brevoortia tyrannus*	8	14.19	3.66
Mullet *Mugil cephalus*	10	13.52	1.46
Sea trout *Cynoscion nebulosus*	9	12.53	2.66
Sheepshead *Archosargus probatocephalus*	7	11.67	1.80
Cutlass *Trichiurus lepturus*	3	11.03	1.86
Group II:			
Sea bass *Centropristus striatus*	5	9.72	2.78
Pinfish *Lagodon rhomboides*	24	9.30	0.91
Croaker *Micropogon undulatus*	8	9.04	4.15
Pigfish *Orthopristis chrysopterus*	15	8.95	1.11
Silver perch *Bairdiella chrysura*	5	8.51	1.27
Spot *Leiostomus xanthurus*	13	7.78	1.41
Group III:			
Flounder *Paralichthys dentatus*	3	6.96	0.74
Spiny boxfish *Chilomycterus schoepfii*	1	6.69	—
Tongue fish *Symphurus plagiusa*	1	6.40	—
Lizard fish *Synodus faetens*	2	6.01	0.01
Toadfish *Opsanus tau*	13	5.57	1.16
Hogchocker *Achirus fasciatus*	1	5.14	—

dent, the fishes are arbitrarily divided into three groups for convenience of discussion. Group I consists of those species which are the most active and are characterized by constant swimming movements. In Group II are those which seem to exhibit less activity than Group I, but are distinctly more active than the bottom-dwelling species of Group III.

Caution should be exercised in viewing too strictly the exact relationship of a few species because of the small number of determinations, especially in the case of the spiny boxfish which is not a typical bottom-dwelling species. However, these clearly demonstrated the tendency of the more sluggish fishes to have a slower rate of oxygen consumption of brain breis and therefore should be included. That the variation of metabolic rate of brain tissue was greater for certain species than for others is evident from the standard deviation obtained. Data for the croaker had a large standard deviation, whereas the standard deviation was small in the case of the pinfish.

When the weight of the pinfish (range 14.5–50 grams) was charted against the rate of oxygen consumption of brain tissue, no correlation was apparent. Similarly, when the length of the toadfish (range 12–40 centimeters) was plotted against the rate of oxygen consumption, no positive correlation was noted. Therefore, it would seem that within the size range of the fish used in this study, the rate of oxygen consumption of brain brei was independent of the weight or the length of the individual, and that the brain tissue of the younger or smaller individual of a species generally consumed about the same amount of oxygen per unit weight as the older or larger specimens.

The results of the brain tissue water-content determinations of 7 species of marine fishes are shown in Table II. In general, the water content is similar in all of the species examined.

TABLE II

Brain tissue water content of 7 species of marine fishes

Species	No. of determinations	Average	Range
Mullet	3	79.02%	77.05–80.90%
Pigfish	3	77.62%	76.33–79.12%
Pinfish	8	79.81%	76.92–82.18%
Sea bass	2	77.08%	76.74–77.42%
Sheepshead	2	76.28%	75.08–77.48%
Spot	7	78.58%	75.00–80.83%
Toadfish	4	78.27%	77.50–79.12%

DISCUSSION

From the results of this study, a positive correlation is evident between the activity of marine teleost fishes and the oxygen consumption of brain breis.

Due to geographical difference in the distribution and relative abundance of fishes during the summer months at Beaufort, N. C., where the present study was made, and Woods Hole, Mass., where most of the early work reported in the literature was done, it is not possible to compare every species, but some species are common to both locations.

Hall and Gray (1929) and Gray and Hall (1930) found that those fishes which had the highest hemoglobin concentration and highest blood sugar level were considered to be the most active. Only two species of fishes investigated by them were used in the present investigation, the menhaden and the toadfish. The menhaden had a higher hemoglobin concentration and blood sugar level than the toadfish. In the present study, the rate of oxygen consumption of excised menhaden brain was distinctively higher than that of the toadfish. Dawson (1933) found the menhaden, as well as other active fishes, to have a greater number of immature circulating erythrocytes than the more sluggish forms, including the toadfish.

In respect to respiratory studies involving marine fishes, Hall and Gray (1929) point out that the menhaden has a higher rate of oxygen consumption than the more sluggish toadfish. Gray (1947) found that the menhaden has about 10 times more gill area than the toadfish per gram of body weight and 15 times more gill area per square centimeter of body surface. A comparison of other species shows that in general a rating of activity based on gill area per gram of weight or per square centimeter of body surface (Gray, unpublished) would agree with an activity rating based on oxygen consumption of brain tissue. A notable exception is the spiny boxfish but as stated previously only one determination was made on this species and more tests may alter the relative rating of this form.

Thus, when comparing the results of these various physiological indices of activity, as reported by other workers, with the results of the present study, it may be noted that there is a positive relationship between the various indices of activity and the data of this paper.

In general the results of the rate of brain tissue oxygen consumption in the present paper are similar to those values obtained by other workers (Fuhrman *et al.*, 1944; Peiss and Field, 1950; and Freeman, 1950). The influence of thermal acclimatization on the rate of oxygen consumption of brain tissue of fish was studied by Peiss and Field (1950) and Freeman (1950). They reported that at a common temperature the brain metabolism was highest for fish acclimatized at the lowest temperatures. No marked variation in respiration data was observed when comparing results of tests conducted in June with later determinations, as is evident in the case of the pinfish. The 24 determinations on this species were made at scattered intervals in June, July and August. The maximum-minimum water temperature taken in the Beaufort Channel from June 17 until August 20, 1952 showed an increase from readings of 80°–79° F. on June 17 to a high of 90°–87° F. on June 28. This was followed by a period of temperature decline and on July 5 the respective readings were 82°–76° F. During the month of August the highest reading was 84° F. and the lowest was 80° F. Thus it would seem that within the narrow temperature range at Beaufort during the period of this investigation no acclimatization effect of brain tissue was observed.

Although some workers have noted a decrease of tissue respiration with increase of body weight (Kayser, Le Breton and Schaeffer, 1925, brains of rats, pigeons and fowls; Hawkins, 1928, rat liver slices; Field *et al.*, 1937, rabbit lens; and Weymouth *et al.*, 1944, mid-gut gland of kelp crab), a similar relationship was not noted in the case of the pinfish and toadfish where the Q_{O_2} appeared to be independent of body weight and body length. However, Grafe (1925) and Terroine and Roche (1925) found that any differences in tissue metabolism of large and

small homoeothermic animals disappear when the tissues are removed from the body of the animal. Recently, Krebs (1950) reported that there is not a simple correlation between body size and Q_{O_2} within the same species, and, in general, tissues of larger species have lower Q_{O_2} values than homologous values of smaller species.

Summary

1. The oxygen consumption of brain tissue of 17 species of marine teleost fishes was determined at 30° C.

2. A positive correlation exists between rate of oxygen consumption and activity of fishes. The more active fishes, like the menhaden, had a higher Q_{O_2} than the sluggish fishes, such as the toadfish.

3. In general, when comparing results of this paper with various physiological indices of activity, a positive relationship was noted.

4. Q_{O_2} of brain tissue of the pinfish and the toadfish were independent of body weight and body length.

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